AD_____

Award Number: DAMD17-01-1-0428

TITLE: The Effects of Folate on the Development of Breast Cancer in a Chemical Rodent Model of Mammary Carcinogenesis

PRINCIPAL INVESTIGATOR: Young-In Kim, M.D.

CONTRACTING ORGANIZATION: University of Toronto
Toronto, Ontario M5S 1A1 Canada

REPORT DATE: August 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | August 2002 | Annual (1 Aug 01 - 31 Jul 02) |

**4. TITLE AND SUBTITLE**
The Effects of Folate on the Development of Breast Cancer in a Chemical Rodent Model of Mammary Carcinogenesis

**5. FUNDING NUMBERS**
DAMD17-01-1-0428

**6. AUTHOR(S)**
Young-In Kim, M.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Toronto
Toronto, Ontario M5S 1A1 Canada

E-Mail: youngin.kim@utoronto.ca

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**



**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. Abstract** *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Epidemiological studies suggest that dietary folate intake and blood levels of folate are inversely related to breast cancer risk. Because only few modifiable risk factors for breast cancer exist, the role of folate in modifying breast cancer risk merits further consideration. Folate is an ideal agent for chemoprevention of breast cancer. It is a natural vitamin, inexpensive, virtually free of side effects, and possesses biologically plausible mechanisms for cancer prevention. However, folate appears to possess dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention. Folate deficiency has an inhibitory, whereas folate supplementation has a promoting, effect on progression of established neoplasms. By contrast, folate deficiency in normal tissues predisposes them to neoplastic transformation, and modest levels of folate supplementation suppress, whereas supraphygiologic doses enhance, the development of tumors in normal tissues. Therefore, the potential effect of folate chemoprevention needs to be clearly established in appropriate animal models before folate supplementation can be considered in humans. Given these considerations, this proposal investigates the effects of dietary folate deficiency and supplementation on mammary tumorigenesis and potential molecular and cellular mechanisms by which folate modulates mammary tumorigenesis in the well established carcinogen rat model of breast cancer.

**14. SUBJECT TERMS**
breast cancer, prevention, nutrition/vitamins/folate, women's health issue, animal model, DNA methylation, DNA damage/repair, mutations

**15. NUMBER OF PAGES**
61

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## Table of Contents

## INTRODUCTION

Breast cancer is the most common cancer and the second leading cause of cancer deaths in women in the United States (*1*). Genetic predisposition (*2-4*) and hormonal/reproductive factors (*5-7*), two important determinants of breast cancer risk, are not readily modifiable. Therefore, much effort has been directed towards identifying potentially modifiable dietary and lifestyle factors that would lead to the prevention of breast cancer. Although epidemiological and animal studies have suggested that dietary factors, such as fat, fiber, vegetables and fruits, antioxidants and alcohol, may influence breast cancer risk, effects of these factors on breast cancer risk are inconsistent and contradictory (*1*). As such, prevention of breast cancer through dietary modifications remains an elusive and challenging task.

Folate, a water-soluble B-vitamin and important co-factor in one-carbon metabolism, has recently been identified as an important nutritional factor that may modulate carcinogenesis (*8-10*). The role of folate in carcinogenesis has been best studied for colorectal cancer (*8-10*). The majority of over 25 published epidemiological studies indicate that dietary folate intake and blood folate levels are inversely associated with colorectal cancer risk (*8-10*). Collectively, these studies suggest an ~40% reduction in the risk of colorectal neoplasms in subjects with highest dietary folate intake compared with those with the lowest intake (*8-10*). These studies also suggest that a modest reduction in folate status is sufficient to enhance colorectal cancer risk (*8-10*). Animal studies have also been generally supportive of a causal relationship between folate depletion and colorectal cancer risk as well as a dose-dependent protective effect of modest levels of dietary folate supplementation (4-10X) above the basal dietary requirement on the development and progression of colorectal neoplasms (*11-15*). Animal studies have also shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels (*12, 16, 17*) and folate intervention after microscopic neoplastic foci are established in the colorectal mucosa (*13, 14*) promote, rather than suppress, colorectal carcinogenesis. An accumulating body of evidence suggests that folate status may also play a modulatory role in the development of several other malignancies (e.g. lung, pancreas, stomach, cervix, esophagus, brain and leukemia) (*8-10*). The precise nature and magnitude of the relationship between folate status and the risk of these malignancies, however, are less clearly defined compared with colorectal cancer.

The relationship between folate status and breast cancer risk has just begun to be reported in the epidemiological literature. Among dietary factors implicated in the development of breast cancer, the inverse relationship between the consumption of vegetables and fruits (the major source of dietary folate) and breast cancer risk (*18*) and the positive correlation between the intake of alcohol (folate antagonist) and breast cancer risk (*19, 20*) have been most consistent (*1*). Among 9 published case-control studies that investigated the relationship between dietary folate intake and breast cancer risk, 7 showed either a significant or equivocal inverse relationship that was not statistically significant, that became nonsignificant after adjustment, or that could not be distinguished from other factors in their relation to risk (*21-27*), whereas 2 showed an unequivocal null association (*28, 29*). In some studies, the observed inverse association was further modified by the intake of alcohol and other folate cofactors (e.g. methionine, vitamins $B_6$ and $B_{12}$) (*24, 26, 27*). One nested case-control study, using stored serum samples, found no association between serum folate and breast cancer risk (*30*). Two large prospective studies have shown a weak inverse association between the total or dietary intake of folate and breast cancer risk (*31, 32*). These prospective studies, however, have indicated that

low intakes of folate increase, whereas high intakes of folate decrease, breast cancer risk among women who regularly consume alcohol (*31, 32*), supporting folate-alcohol interactions in breast carcinogenesis observed in case-control studies (*24, 26, 27*).

Two animal studies published to date have produced conflicting results concerning the effect of folate on mammary tumorigenesis. In mice with confirmed spontaneous mammary cancers, daily intravenous injections of fermentation *Lactobacillus casei* factor (pteroyltriglutamate) significantly regressed mammary tumors and decreased new mammary tumor formation and lung metastases (*33*). Another study employing the *N*-methyl-*N*-nitrosourea (MNU) rat model showed that a folate-deficient diet provided during the initiation phase of mammary tumorigenesis significantly reduced tumor multiplicity and increased tumor latency compared with a control and folate-supplemented diet (*34*). The incidence of mammary tumors, however, was not significantly different among these groups (*34*). Several inherent limitations associated with these animal studies, however, preclude a definitive conclusion concerning the effect of folate on mammary tumorigenesis.

Because only few modifiable risk factors for breast cancer exist, recent epidemiological observations which suggest that folate deficiency increases, whereas supplementation reduces, breast cancer risk merit further consideration. Folate is an ideal agent for potential chemoprevention of breast cancer. It is a natural vitamin, inexpensive, virtually free of side effects (*35*), and possesses biologically plausible mechanisms for cancer prevention (*8-10*). However, the results from published epidemiological and animal studies have been neither consistent nor convincing. Furthermore, a growing body of evidence suggests that folate possesses the dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention (*11-17*). Folate deficiency has an inhibitory, whereas folate supplementation has a promoting, effect on progression of established neoplasms (*11-17*). By contrast, folate deficiency in normal epithelial tissues appears to predispose them to neoplastic transformation, and modest levels of folate supplementation suppress the development of tumors in normal tissues (*11-17*). Therefore, the potential effect of folate chemoprevention needs to be clearly elucidated in appropriate animal models before folate supplementation can be considered in humans. Given these considerations, we proposed to study the effects of dietary folate deficiency and supplementation on the development and progression of mammary tumors in the well-established MNU rat model of breast cancer in three animal experiments and to investigate potential molecular mechanisms by which dietary folate modulates mammary tumorigenesis. Notwithstanding the limitations associated with animal models, the MNU rat model is widely used to determine the effects of dietary factors on mammary tumorigenesis for the following reasons: (a) histological similarities of adenocarcinomas to human breast cancer; (b) local invasiveness and metastatic potential; (c) a clear operational distinction between the initiation and promotion stages; and (d) hormonally dependent mammary tumorigenesis (*36-40*).

## BODY

**Task 1 (Specific Aim I):** To determine whether sustained folate deficiency of a moderate degree enhances, and whether a modest degree of folate supplementation above the basal requirement suppresses, the development of mammary tumors in the MNU rat model of mammary carcinogenesis (*initiation + promotion combined*)

a. Animal experiment (feeding + MNU injectIon + sacrifice + sample harvesting) [months 1 – 8]
b. Preparation of samples (paraffin embedding, preparation of slides and staining of tumors and adjacent normal tissues, DNA and RNA extraction, tissue folate extraction) [months 8 – 9]
c. Histologic analysis of tumors, microdissection of neoplastic foci and adjacent normal tissues for DNA extraction, folate analysis [months 9 – 11]
d. Data analysis [months 11 – 12]

During the first year of funding (August 1, 2001 – July 31, 2002), we investigated the effect of dietary folate deficiency and supplementation on the development and progression of mammary tumors in the MNU rat model. Weaning, female Sprague-Dawley rats were fed diets containing either 0 mg (deficient; n=22), 2 mg (basal dietary requirement, control; n=20) or 8 mg (supplemented; n=20) folate /kg diet for 30 weeks. At 50 days of age, rats received an intraperitoneal injection of MNU (50 mg/kg body weight). Body weights were recorded weekly. The daily food consumption of each group was measured on a predetermined day of each week. All rats were palpated for mammary tumors once a week beginning 4 weeks after MNU administration. The number, size and location of each tumor were recorded in a manner that, after histological diagnosis, the time of appearance of the cancers could be determined. All the rats were monitored daily for clinical evidence of illness or morbidity and those approaching a moribund state were promptly euthanized. In addition, rats with tumor burden exceeding 10% of body weight, tumors >15-20 mm in diameter, tumors that impaired normal movement of the animals, and ulcerating tumors were immediately euthanized during the study. Blood was collected from the tail of each rat within a week of MNU injection and from the heart at necropsy for the serum folate assay. Given the latency period of 3-6 months associated with a single intraperitoneal MNU injection and the average duration for the systemic and tissue folate levels to stabilize, the rats were sacrificed by carbon dioxide inhalation followed by cervical dislocation at 23 weeks after MNU injection (27 weeks after dietary intervention or 30 weeks of age). The liver from each rat was harvested for hepatic folate concentration determination. All macroscopic mammary tumors were counted, excised and weighed, and diameters of each tumor were measured using a digital caliper for final tumor volume computation in a blinded fashion. One-half of each macroscopic tumor was processed for DNA extraction. The other half of the tumor was processed in a standard manner for histological analysis according to Russo et al. (*39*) by three experienced pathologists blinded to the study group independently. In the case of a discrepancy, two similar interpretations were utilized for the final analysis. Normal mammary tissue was processed for DNA extraction and mammary tissue folate determination. Between-group comparisons of continuous variables were assessed using the Kruskal-Wallis and Mann-Whitney non-parametric tests. For categorical response variables, differences among the groups were assessed by Pearson chi-square test. The Kaplan-Meier survival analysis and the

Log Rank test were used to compare the rates of tumor appearance among the three groups. All significance tests were two sided and were considered statistically significant if the observed significance level was <0.05. Results are expressed as mean ± SEM. Statistical analyses were performed using SPSS (version 10).

Serum folate concentrations accurately reflected dietary folate levels at the time of MNU administration and at necropsy (P<0.001). The mean folate concentrations of the normal mammary glad of the folate-deficient group were significantly lower than those of the control and folate-supplemented groups (P<0.001), whereas no significant differences between the control and folate-supplemented groups was obseved. The final incidence of mammary tumors in the folate-deficient group was significantly lower than that of the control and folate-supplemented groups (55% versus 90% and 75%, respectively, P=0.04). Kaplan-Meier analyses also demonstrated similar cumulative tumor incidence trends (P=0.06). By contrast, dietary folate supplementation did not significanlty modulate both the final and cumulative incidences of mammary tumors compared with the control gropu. Dietary folate status had no significant effect on mean volume, weight, latency or multiplicity of mammary tumors. These data suggest that dietary folate deficiency of a moderate degree suppresses mammary tumorigenesis in this model. By contrast, dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis.

In summary, all specific tasks outlined in Task 1 have been completed. One abstract arising from Task 1 entitled "Dietary folate deficiency suppresses mammary tumorigenesis in a chemical carcinogen rat model of breast cancer" will be presented in Poster Session: Chemoprevention, P13-8 at the Era of Hope meeting in Orlando, Florida on September 26, 2002 (**Appendix 1**). One manuscript arising from Task 1 entitled "Effects of dietary folate on N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats" has been prepared and submitted to Cancer Research (**Appendix 2**).

**Task 2 (Specific Aim II):** To determine whether folate deficiency enhances, and whether folate supplementation suppresses, the *initiation* of mammary carcinogenesis

a. Animal experiment (feeding + MNU injection + sacrifice + sample harvesting) [months 4 – 11]
b. Preparation of samples (paraffin embedding, preparation of slides and staining of tumors and adjacent normal tissues, DNA and RNA extraction, tissue folate extraction) [months 11 – 12]
c. Histologic analysis of tumors, microdissection of neoplastic foci and adjacent normal tissues for DNA extraction, folate analysis [months 12 – 14]
d. Data analysis [months 14 – 15]

During the first year of funding (August 1, 2001 – July 31, 2002), we investigated the effect of dietary folate deficiency and supplementation on the *initiation* phase of mammary tumors in the MNU rat model. Weaning, female Sprague-Dawley rats were fed diets containing either 0 mg (deficient; n=21), 2 mg (basal dietary requirement, control; n=20) or 8 mg (supplemented; n=20) folate /kg diet. At 50 days of age, rats received an intraperitoneal injection of MNU (50 mg/kg body weight). The initial diets were terminated one week following

MNU-injection and all the rats were placed on the control (2 mg folate/kg diet) diet until the time of sacrifice (30 weeks of age). Body weights were recorded weekly. The daily food consumption of each group was measured on a predetermined day of each week. All rats were palpated for mammary tumors once a week beginning 4 weeks after MNU administration. The number, size and location of each tumor were recorded in a manner that, after histological diagnosis, the time of appearance of the cancers could be determined. All the rats were monitored daily for clinical evidence of illness or morbidity and those approaching a moribund state were promptly euthanized. In addition, rats with tumor burden exceeding 10% of body weight, tumors >15-20 mm in diameter, tumors that impaired normal movement of the animals, and ulcerating tumors were immediately euthanized during the study. Blood was collected from the tail of each rat within a week of MNU injection and from the heart at necropsy for the serum folate assay. Given the latency period of 3-6 months associated with a single intraperitoneal MNU injection and the average duration for the systemic and tissue folate levels to stabilize, the rats were sacrificed by carbon dioxide inhalation followed by cervical dislocation at 23 weeks after MNU injection (27 weeks after dietary intervention or 30 weeks of age). The liver from each rat was harvested for hepatic folate concentration determination. All macroscopic mammary tumors were counted, excised and weighed, and diameters of each tumor were measured using a digital caliper for final tumor volume computation in a blinded fashion. One-half of each macroscopic tumor was processed for DNA extraction. The other half of the tumor will be processed in a standard manner for histological analysis according to Russo et al. (*39*) by three experienced pathologists blinded to the study group independently. In the case of a discrepancy, two similar interpretations will be utilized for the final analysis. Normal mammary tissue will be processed for DNA extraction and mammary tissue folate determination. Between-group comparisons of continuous variables will be assessed using the Kruskal-Wallis and Mann-Whitney non-parametric tests. For categorical response variables, differences among the groups will be assessed by Pearson chi-square test. The Kaplan-Meier survival analysis and the Log Rank test will be used to compare the rates of tumor appearance among the three groups. All significance tests are two sided and were considered statistically significant if the observed significance level was $<0.05$. Results are expressed as mean ± SEM. Statistical analyses will be performed using SPSS (version 10).

We have completed tasks (a) and (b) of Task 2 as of July 21, 2002 and will complete tasks (c) and (d) over the next 2-3 months.

**Task 3 (Specific Aim III):** To determine whether folate deficiency enhances, and whether folate supplementation suppresses, the *promotion* of mammary carcinogenesis

e. Animal experiment (feeding + MNU injection + sacrifice + sample harvesting) [months 7 – 14]
f. Preparation of samples (paraffin embedding, preparation of slides and staining of tumors and adjacetn normal tissues, DNA and RNA extraction, tissue folate extraction) [months 14 – 15]
g. Histologic analysis of tumors, microdissection of neoplastic foci and adjacent normal tissues for DNA extraction, folate analysis [months 15 – 17]
h. Data analysis [months 17 – 18]

During the first year of funding (August 1, 2001 – July 31, 2002), we investigated the effect of dietary folate deficiency and supplementation on the *promotion* phase of mammary tumors in the MNU rat model. Weaning, female Sprague-Dawley rats (n=93) were placed on the control diet (2 mg folate/kg diet). At 50 days of age, rats received an intraperitoneal injection of MNU (50 mg/kg body weight). One week following MNU administration, rats were randomized to receive diets containing either 0 mg (deficient; n=33), 2 mg (basal dietary requirement, control; n=30) or 8 mg (supplemented; n=30) folate /kg diet until the time of sacrifice (30 weeks of age). Body weights were recorded weekly. The daily food consumption of each group was measured on a predetermined day of each week. All rats were palpated for mammary tumors once a week beginning 4 weeks after MNU administration. The number, size and location of each tumor were recorded in a manner that, after histological diagnosis, the time of appearance of the cancers could be determined. All the rats were monitored daily for clinical evidence of illness or morbidity and those approaching a moribund state were promptly euthanized. In addition, rats with tumor burden exceeding 10% of body weight, tumors >15-20 mm in diameter, tumors that impaired normal movement of the animals, and ulcerating tumors were immediately euthanized during the study. Blood was collected from the tail of each rat within a week of MNU injection and will be collected from the heart at necropsy for the serum folate assay. Given the latency period of 3-6 months associated with a single intraperitoneal MNU injection and the average duration for the systemic and tissue folate levels to stabilize, the rats will be sacrificed by carbon dioxide inhalation followed by cervical dislocation at 23 weeks after MNU injection (27 weeks after dietary intervention or 30 weeks of age). The liver from each rat will be harvested for hepatic folate concentration determination. All macroscopic mammary tumors will be counted, excised and weighed, and diameters of each tumor will be measured using a digital caliper for final tumor volume computation in a blinded fashion. One-half of each macroscopic tumor will be processed for DNA extraction. The other half of the tumor will be processed in a standard manner for histological analysis according to Russo et al. (*39*) by three experienced pathologists blinded to the study group independently. In the case of a discrepancy, two similar interpretations will be utilized for the final analysis. Normal mammary tissue will be processed for DNA extraction and mammary tissue folate determination. Between-group comparisons of continuous variables will be assessed using the Kruskal-Wallis and Mann-Whitney non-parametric tests. For categorical response variables, differences among the groups will be assessed by Pearson chi-square test. The Kaplan-Meier survival analysis and the Log Rank test will be used to compare the rates of tumor appearance among the three groups. All significance tests are two sided and were considered statistically significant if the observed significance level was $<0.05$. Results are expressed as mean ± SEM. Statistical analyses will be performed using SPSS (version 10).

We will complete task (a) of Task 3 by the end of October 31, 2002 and tasks (b), (c) and (d) of Task 3 as of January 31, 2003.

**Task 4 (Specific Aim IV):** To determine molecular mechanisms by which folate status modulates mammary tumorigenesis in this model (using samples predominantly from Task 1)

a. Genomic DNA methylation and site-specific CpG-rich promoer methylation (ER, p16, p53 genes) [months 10 – 14]

b. Development of immunochemical staining with anti-methylcytosine antibody [months 10 –12]
c. Immunohistochemical determination of expression of ER, p16, p53 proteins [months 12 –14]
d. RNase proteciton assay to determine steady-state mRNA levels of DNA methyltransferases (Dnmt1, Dnmt3a, Dnmt3b) and demethylase [months 14 – 16]
e. Determination of DNA methyltransferase and demethylase activities [months 16 – 18]
f. Immunohistochemical determination of expression of Dnmt1, Dnmt3a, Dnmt3b and demethylase [months 18 – 20]
g. Genomic and site-specifci p53 DNA strand breaks [months 20 – 22]
h. Microsatellite instability [months 22 – 25]
i. PCR-RFLP/liquid hybridization to detect Ha-ras mutations [months 25 –27]
j. Immunostaining/PCR-SSCP/direct sequencing to detect p53 mutations [months 27 – 29]
k. PCNA and apoptosis [months 29 – 31]
l. Immunochemical detection of cyclin D1 overexpression [months 31 – 32]
m. Data analysis [months 32 – 34]

During the first year of funding (August 1, 2001 – July 31, 2002), we have completed task (a) of Task 4. DNA from normal mammary tissues and mammary tumors was extracted by a standard technique using a lysis buffer containing proteinase K followed by phenol, chloroform, and isoamyl alcohol organic extraction (*41*). The size of DNA estimated by agarose-gel electrophoresis was >20 kb in all instances. No RNA contamination was detected on agarose-gel electrophoresis. The final preparations had a ratio of $A_{260}$ to $A_{280}$ between 1.8 and 2.0. The concentration of each DNA sample was determined as the mean of 3 independent spectrophotometric readings. The methylation status of cytosine-guanine (CpG) sites in genomic DNA from normal mammary tissues and mammary tumors was determined by the in vitro methyl acceptance capacity of DNA using [$^3$H-methyl]S-adenosylmethionine (SAM) as a methyl donor and a prokaryotic CpG DNA methyltransferase, Sss1, as previously described (*12, 14, 42, 43*). The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous [$^3$H-methyl] incorporation. Briefly, mammary tumor and non-neoplastic mammary gland DNA (500 ng) was incubated with 2.0 $\mu$Ci of [$^3$H-methyl]SAM (New England Nuclear, Boston, MA), 3 units of Sss1 methylase (New England Biolabs, Beverly, MA), and 1X Sss1 methylation buffer [120mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM EDTA, 1 mM dithiothreitol] in a total volume of 30 $\mu$L for 1 hour at 30°C. The Sss1 was inactivated by incubating at 65°C for 10 minutes. The in vitro methylated DNA was isolated from a 15 $\mu$l aliquot of the reaction mixture by filtration on a Whatman DE-81 ion-exchange filter (Fisher Scientific, Springfield, NJ). The DNA was washed three times with 0.5 M sodium phosphate buffer (pH 7.0), air-dried and the radioactivity of the DNA retained in the filters was measured by scintillation counting using a nonaqueous scintillation fluor. The amount of radiolabel bound to a filter from an incubation mixture without DNA (control) was used as background and was subtracted from the values obtained with mixtures containing DNA. The background value was always <1% of the uptake observed with DNA samples. All analyses were performed in duplicate. Differences in genomic DNA methylation between normal mammary gland and tumor in each diet group was assessed by the Wilcoxon signed ranks test.

The degree of $^{3}$H-methyl incorporation into DNA of the mammary adenocarcinoma and into DNA from the pair-matched non-neoplastic mammary tissue was not significantly different among the three dietary groups. However, the degree of $^{3}$H-methyl incorporation into DNA of the mammary adenocarcinomas, which is inversely related to the extent of genomic DNA methylation, was 4- to 5-fold higher than that of non-neoplastic mammary tissue within each dietary group, indicating a significantly lower degree of genomic DNA methylation in the adenocarcinomas compared with the normal mammary tissue. DNA methylation is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in chromatin modifications and in the development of mutations (*44, 45*). Neoplastic cells simultaneously harbor widespread genomic DNA hypomethylation and more specific regional areas of hypermethylation (*44, 45*). Genomic and protooncogene-specific hypomethylation appears to be an early, and consistent, event in carcinogenesis (*44, 45*). There appears to be a direct correlation between the extent of genomic DNA hypomethylation and tumor progression (*44, 45*). Genomic hypomethylation results in genomic instability and increased mutations, and protooncogene-specific hypomethylation results in increased gene expression (*44, 45*). In addition, site-specific hypomethylation at the promoter region of tumor suppressor and mismatch repair genes is an important mechanism in gene silencing in carcinogenesis (*44, 45*). Although promoter CpG islands hypermethylation of several genes including *BRCA1, ER, p16, E-Cadherin, TMS1, RASSF1*, leading to inactivation of these genes have been observed in human breast cancer (*46-51*), very few studies have reported genomic hypomethylation in human breast cancer (*52, 53*). To our knowledge, our study is the first to demonstrate that genomic DNA hypomethylation is an epigenetic phenomenon associated with MNU-induced mammary tumorigenesis in rats. The extent of genomic DNA methylation in mammary adenocarcinomas and in nonneoplastic mammary tissues was not significantly modulated by folate status. This observation suggests that altered genomic DNA methylation was not a likely mechanism by which folate deficiency suppressed mammary tumorigenesis in our study. Folate, in the form of 5-methyltetrahydrofolate, is involved in remethylation of homocysteine to methionine, which is a precursor of SAM, the primary methyl group donor for most biological methylation reactions (*8-10*). Although isolated folate deficiency has been shown to induce genomic DNA hypomethylation in circulating lymphocytes in humans (*54, 55*), this effect has not been demonstrated in the colorectum or liver in rodents fed the same folate-deficient diet employed in the present study (*12, 14, 56*). However, an extremely severe degree of folate deficiency has been shown to induce genomic (*42*) and site-specific *p53* (*43*) DNA hypomethylation in rat liver, although other studies have disputed the finding on genomic DNA methylation (*43*). Because both site-specific hypo- and hypermethylation play a role in carcinogenesis (*44, 45*) and because folate may modulate DNA methylation in a site-specific manner (*43*), it would be of great interest to study site-specific methylation of protooncogenes and tumor suppressor genes implicated in mammary tumorigenesis (*2-4*) and the effect of folate status.

The data concerning genomic mammary DNA methylation in MNU-mammary tumorigenesis in rats have been incorporated into a manuscript entitled "Effects of dietary folate on N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats," which has been submitted to Cancer Research (**Appendix 2**). At present, we are working on tasks (b), (h) and (i) of Task 4 and will begin other tasks as outlined above.

## KEY RESEARCH ACCOMPLISHMENTS

1. Our data from Task 1 indicate that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats and that dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis in this model. These observations contradict the generally accepted notion based on epidemiologic evidence, which suggests that folate deficiency enhances, whereas folate supplementation suppresses, the development of breast cancer in humans. Notwithstanding the limitations associated with this model, our data suggest that the role of folate in mammary tumorigenesis needs to be clarified in subsequent animal and human studies for safe and effective prevention of breast cancer.
2. Our study is the first to demonstrate that genomic DNA hypomethylation is an epigenetic phenomenon associated with MNU-induced mammary tumorigenesis. However, the extent of genomic DNA methylation in mammary tumors was not significantly modulated by folate status. This observation suggests that altered genomic DNA methylation was not a likely mechanism by which folate deficiency suppressed mammary tumorigenesis in this study.

## REPORTABLE OUTCOMES

1. Kotsopoulos J, Sohn K-J, Martin R, Renlund R, McKerlie C, Hwang S, Medline A, **Kim YI**. Dietary folate deficiency suppresses mammary tumorigenesis in a chemical carcinogen rat model of breast cancer. (Scheduled for poster presentation at Chemoprevention, P13-8 at Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orlando, Florida, September 25 – 28, 2002; **Appendix 1**)
2. Kotsopoulos J, Sohn K-J, Martin R, Renlund R, McKerlie C, Hwang S, Medline A, **Kim YI**. Effects of dietary folate on N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats. (Submitted to Cancer Research; **Appendix 2**)

## CONCLUSIONS

Our data arising from Task 1 completed during the first year of funding (August 1, 2001 – July 31, 2002) suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats. By contrast, dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis. Notwithstanding the limitations associated with this model, our data suggest that the role of folate in mammaray tumorigenesis needs to be clarified in future studies for safe and effective prevention of breast cancer. In light of the findings from Task 1, further investigation is warranted to elucidate the role of folate in mammary tumorigenesis. The effect of folate on the initiation (Task 2) and promotion/progression (Task 3) phases of MNU-induced tumorigenesis needs to be clearly defined. Lower doses of MNU, lower fat content and higher levels of folate supplementation may be necessary to clearly elucidate the effect of folate on mammary tumorigenesis in this model. The effect of folate on mammary tumorigenesis observed in the present study needs to be confirmed in other animal models. Given the possible interactions of folate with alcohol and other folate co-factors in modifying breast cancer risk observed in some epidemiological studies, these potential interactions merit further investigation. Our data from

Tasks 1 and 4 also indicate that genomic mammary DNA hypomethylation is an epigenetic mechanism by which mammary tumors develop in the MNU rat model. Studies are underway to investigate site and gene-specific DNA methylation changes in this model. Our data also suggest that changes in genomic mammary DNA methylation are not a likely mechanism by which folate modulates mammary tumorigenesis in this model. Studies are underway to investigate other molecular mechanisms as outlined in Task 4.

## REFERENCES


1. World Cancer Research Fund/American Institute for Cancer Research, *Food, nutrition and the prevention of cancer: a global perspective* (The American Institute for Cancer Research, Washington, DC, 1997).
2. D. A. Dillon, C. L. Howe, S. Bosari, J. Costa, *Crit Rev Oncog* **9**, 125-40 (1998).
3. L. W. Ellisen, D. A. Haber, *Annu Rev Med* **49**, 425-36 (1998).
4. A. M. Martin, B. L. Weber, *J Natl Cancer Inst* **92**, 1126-35 (Jul 19, 2000).
5. R. B. Duda, *Compr Ther* **21**, 29-34 (1995).
6. *Lancet* **347**, 1713-27 (Jun 22, 1996).
7. *Lancet* **350**, 1047-59 (Oct 11, 1997).
8. Y. I. Kim, *J Nutr Biochem* **10**, 66-88 (1999).
9. Y. I. Kim, *Nutr Rev* **57**, 314-21 (Oct, 1999).
10. J. B. Mason, S. W. Choi, *Adv Enzyme Regul* **40**, 127-41 (2000).
11. M. L. Cravo *et al.*, *Cancer Res* **52**, 5002-6 (Sep 15, 1992).
12. Y. I. Kim *et al.*, *Gut* **39**, 732-40 (Nov, 1996).
13. J. Song, A. Medline, J. B. Mason, S. Gallinger, Y. I. Kim, *Cancer Res* **60**, 5434-40 (Oct 1, 2000).
14. J. Song *et al.*, *Cancer Res* **60**, 3191-9 (Jun 15, 2000).
15. M. J. Wargovich *et al.*, *Carcinogenesis* **21**, 1149-55 (Jun, 2000).
16. M. J. Wargovich *et al.*, *Cancer Epidemiol Biomarkers Prev* **5**, 355-60 (May, 1996).
17. R. K. Le Leu, G. P. Young, G. H. McIntosh, *Carcinogenesis* **21**, 2261-5 (Dec, 2000).
18. S. Gandini, H. Merzenich, C. Robertson, P. Boyle, *Eur J Cancer* **36**, 636-46 (Mar, 2000).
19. S. A. Smith-Warner *et al.*, *Jama* **279**, 535-40 (Feb 18, 1998).
20. V. Bagnardi, M. Blangiardo, C. L. Vecchia, G. Corrao, *Br J Cancer* **85**, 1700-5 (Nov 30, 2001).
21. S. Graham *et al.*, *Am J Epidemiol* **134**, 552-66 (Sep 15, 1991).
22. J. L. Freudenheim *et al.*, *J Natl Cancer Inst* **88**, 340-8 (Mar 20, 1996).
23. A. Ronco *et al.*, *Nutr Cancer* **35**, 111-9 (1999).
24. T. E. Rohan, M. G. Jain, G. R. Howe, A. B. Miller, *J Natl Cancer Inst* **92**, 266-9 (Feb 2, 2000).
25. F. Levi, C. Pasche, F. Lucchini, C. La Vecchia, *Int J Cancer* **91**, 260-3 (Jan 15, 2001).
26. E. Negri, C. La Vecchia, S. Franceschi, *J Natl Cancer Inst* **92**, 1270-1 (Aug 2, 2000).
27. M. J. Shrubsole *et al.*, *Cancer Res* **61**, 7136-41 (Oct 1, 2001).
28. B. Thorand, L. Kohlmeier, N. Simonsen, C. Croghan, M. Thamm, *Public Health Nutr* **1**, 147-56 (Sep, 1998).
29. N. Potischman *et al.*, *Int J Cancer* **82**, 315-21 (Jul 30, 1999).
30. K. Wu *et al.*, *Cancer Epidemiol Biomarkers Prev* **8**, 209-17 (Mar, 1999).
31. S. Zhang *et al.*, *Jama* **281**, 1632-7 (May 5, 1999).
32. T. A. Sellers *et al.*, *Epidemiology* **12**, 420-8 (Jul, 2001).
33. R. Lewisohn, C. Leuchtenberger, R. Leuchtenberger, J. C. keresztesy, *Science* **104**, 436-7 (1946).
34. J. E. Baggott *et al.*, *J Natl Cancer Inst* **84**, 1740-4 (Nov 18, 1992).
35. N. R. Campbell, *Arch Intern Med* **156**, 1638-44 (Aug 12-26, 1996).
36. D. P. Rose, B. Pruitt, P. Stauber, E. Erturk, G. T. Bryan, *Cancer Res* **40**, 235-9 (Feb, 1980).

37. D. L. McCormick, C. B. Adamowski, A. Fiks, R. C. Moon, *Cancer Res* **41**, 1690-4 (May, 1981).
38. H. J. Thompson, H. Adlakha, *Cancer Res* **51**, 3411-5 (Jul 1, 1991).
39. J. Russo, I. H. Russo, A. E. Rogers, M. J. Van Zwienten, B. Gusterson, in *Pathology of tumours in laboratory animals* V. Turusov, U. Mohr, Eds. (IARC Scientific Publications, Lyon, 1990), vol. 1, pp. 47-78.
40. I. H. Russo, J. Russo, *Environ Health Perspect* **104**, 938-67 (Sep, 1996).
41. P. W. Laird *et al.*, *Nucleic Acids Res* **19**, 4293 (Aug 11, 1991).
42. M. Balaghi, C. Wagner, *Biochem Biophys Res Commun* **193**, 1184-90 (Jun 30, 1993).
43. Y. I. Kim *et al.*, *Am J Clin Nutr* **65**, 46-52 (Jan, 1997).
44. S. B. Baylin, J. G. Herman, J. R. Graff, P. M. Vertino, J. P. Issa, *Adv Cancer Res* **72**, 141-96 (1998).
45. P. A. Jones, P. W. Laird, *Nat Genet* **21**, 163-7 (Feb, 1999).
46. Y. L. Ottaviano *et al.*, *Cancer Res* **54**, 2552-5 (May 15, 1994).
47. J. C. Rice, K. S. Massey-Brown, B. W. Futscher, *Oncogene* **17**, 1807-12 (Oct 8, 1998).
48. J. R. Graff *et al.*, *Cancer Res* **55**, 5195-9 (Nov 15, 1995).
49. S. A. Foster, D. J. Wong, M. T. Barrett, D. A. Galloway, *Mol Cell Biol* **18**, 1793-801 (Apr, 1998).
50. D. G. Burbee *et al.*, *J Natl Cancer Inst* **93**, 691-9 (May 2, 2001).
51. K. E. Conway *et al.*, *Cancer Res* **60**, 6236-42 (Nov 15, 2000).
52. J. Soares *et al.*, *Cancer* **85**, 112-8 (Jan 1, 1999).
53. J. Bernardino *et al.*, *Cancer Genet Cytogenet* **97**, 83-9 (Sep, 1997).
54. G. C. Rampersaud, G. P. Kauwell, A. D. Hutson, J. J. Cerda, L. B. Bailey, *Am J Clin Nutr* **72**, 998-1003 (Oct, 2000).
55. R. A. Jacob *et al.*, *J Nutr* **128**, 1204-12 (Jul, 1998).
56. Y. I. Kim *et al.*, *Am J Clin Nutr* **61**, 1083-90 (May, 1995).

## APPENDICES

1. Kotsopoulos J, Sohn K-J, Martin R, Renlund R, McKerlie C, Hwang S, Medline A, **Kim YI**. Dietary folate deficiency suppresses mammary tumorigenesis in a chemical carcinogen rat model of breast cancer. (Scheduled for poster presentation at Chemoprevention, P13-8 at Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orlando, Florida, September 25 – 28, 2002)
2. Kotsopoulos J, Sohn K-J, Martin R, Renlund R, McKerlie C, Hwang S, Medline A, **Kim YI**. Effects of dietary folate on N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats. (Submitted to Cancer Research)

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
1077 PATCHEL STREET
FORT DETRICK, MD 21702-5024

REPLY TO
ATTENTION OF:

# Please note the following:

Deficiency

The abstract entitled: **DIETARY FOLATE SUPPRESSES MAMMARY TUMORIGENESIS IN A CHEMICAL CARCINOGEN RAT MODEL OF BREAST CANCER**

for contract: **DAMD170110428**

to be presented by: **Young-In Kim**

at the September 25–28, 2002, Era of Hope meeting will be presented in Poster Session: **Chemoprevention, P13-8**

that is scheduled for: **Thursday, September 26, 2002, at 6:30-8:30 p.m.**

**Poster Session Setup:**
**September 25, 2002, from 1:00 p.m. – 6:00 p.m.**
**September 26, 2002, from 7:00 a.m. – 3:00 p.m.**

**During designated Poster Session periods, Principal Investigators, or designated representatives, will be required to be at their posters to discuss their work.**

**Posters must be taken down by 10:00 a.m. on September 29, 2002.**

0207013

# DIETARY FOLATE DEFICIENCY SUPPRESSES MAMMARY TUMORIGENESIS IN A CHEMICAL CARCINOGEN RAT MODEL OF BREAST CANCER

**Joannae Kotsopoulos, Kyoung-Jin Sohn, Rochelle Martin, Richard Renlund, Colin McKerlie, Stephen Hwang, Alan Medline, Young-In Kim**

Departments of Laboratory Medicine & Pathobiology, Medicine and Nutritional Sciences, University of Toronto

Youngin.kim@utoronto.ca

Epidemiological studies have suggested that dietary folate intake is inversely related to the risk of breast cancer. This study investigated the effect of dietary folate on the development and progression of N-methyl-N-nitrosourea (MNU)-induced mammary tumorigenesis in rats. Weanling, female Sprague-Dawley rats were randomized to receive an amino acid-defined diet containing either 0 mg (moderately folate deficient; n=22), 2 mg (basal dietary requirement [control]; n=20) or 8 mg (supplemented; n=20) folate/kg diet. At 50 days of age, all the rats received an intraperitoneal injection of MNU (50 mg/kg body weight) and the initial dietary interveniton was continued for additional 23 weeks. At necropsy, all macroscopic mammary tumors were identified and histologically confirmed for adenocarcinoma or its precursor, adenoma. Serum folate concentrations accurately reflected dietary folate levels at the time of MNU administration and at necropsy ($P<0.001$). The mean folate concentrations of the normal mammary gland of the folate-deficient group were significantly lower than those of the control and folate-supplemented groups ($P<0.001$), whereas no significant difference between the control and folate-supplemented groups was observed. The final incidence of mammary tumors in the folate-deficient group was significantly lower than that of the control and folate-supplemented groups (55% versus 90% and 75%, respectively, $P=0.04$). Kaplan-Meier analyses also demonstrated similar cumulative tumor incidence trends ($P=0.06$). By contrast, dietary folate supplementation did not significantly modulate both the final and cmulative incidences of mammary tumors compared with the control group. Dietary folate status had no significant effect on mean volume, weight, latency or multiplicity of mammary tumors. These data suggest that dietary folate deficiency of a moderate degree suppresses mammary tumorigenesis in this model. By contrast, dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary tumorigenesis. The role of folate in mammaray tumorigenesis needs to be clarified in future studies for safe and effective prevention of breast cancer.

The U.S. Army Medical Research Materiel Command under DAMD17-01-1-0428 supported this work.

# Effects of dietary folate on N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats[1]

Joanne Kotsopouolos, Kyoung-Jin Sohn, Rochelle Martin, Richard Renlund, Colin Mckerlie, Stephen W. Hwang, Alan Medline, Young-In Kim[2]

Department of Nutritional Sciences, University of Toronto, Toronto, Ontario, Canada (J.K., Y-I.K.)
Department of Medicine, University of Toronto, Toronto, Ontario, Canada (K-J.S., S.W.H., Y-I.K.)
Inner City Health Research Unit, St. Michael's Hospital, Toronto, Ontario, Canada (R.M., S.W.H.)
Division of Comparative Medicine, University of Toronto, Toronto, Ontario, Canada (R.R.)
Integrative Biology Research Program, Hospital for Sick Children, Toronto, Ontario, Canada (C.M.)
Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada (A.M.)
Division of Gastroenterology, Department of Medicine, St. Michael's Hospital, Toronto, Ontario, Canada (Y-I.K.)

**Running Title:** Folate and breast cancer

**Key Words:** Folate, breast cancer, *N*-methyl-*N*-nitrosourea, DNA methylation, rats

1. This project has been supported in part by the Department of Defense Breast Cancer Research Program of the US Army Medical Research and Materiel Command's Office (Award Number DAMD17-01-1-0428; to Y-I.K.) and by the Canadian Institutes of Health Research (to Y-I.K.). Y-I.K. is a recipient of a scholarship from the Canadian Institutes of Health Research. The content of this publication does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred.

2. To whom requests for reprints should be addressed, at Room 7258, Medical Sciences Building, University of Toronto, 1 King's College Circle, Toronto, Ontario, Canada, M5S 1A8; Telephone: 416-978-1183; Fax: 416-978-8765; e-mail: youngin.kim@utoronto.ca

3. The abbreviations used are: CpG, cytosine-guanine dinucleotides; FPGS, folylpolyglutamate synthetase; MNU, *N*-methyl-*N*-nitrosourea; and SAM, S-adenosylmethionine.

## ABSTRACT

Epidemiological studies have suggested that dietary folate intake is inversely related to breast cancer risk. However, epidemiological evidence has not been consistent nor has it provided unequivocal support for this purported inverse relationship. This study investigated the effect of dietary folate on N-methyl-N-nitrosourea (MNU)-induced mammary tumorigenesis in rats. Weanling, female Sprague-Dawley rats were fed diets containing either 0 mg (deficient; n=22), 2 mg (basal dietary requirement, control; n=20) or 8 mg (supplemented; n=20) folate/kg diet for 30 weeks. At 50 days of age, rats received an intraperitoneal injection of MNU (50 mg/kg body weight). At necropsy, all macroscopic mammary tumors were identified and examined microscopically. The effect of dietary folate on genomic DNA methylation in mammary tumorigenesis was determined by the in vitro methyl acceptance assay. The incidence of mammary adenomas and adenocarcinomas in the folate-deficient group was significantly lower than that of the control and folate-supplemented groups (55% versus 90% and 75%, respectively, P=0.043). Kaplan-Meier analyses also demonstrated a similar trend in the rates of appearance of either adenomas or adenocarcinomas (P=0.06). By contrast, folate supplementation did not significantly modulate mammary tumorigenesis compared with the control group. Although mammary tumors were significantly hypomethylated compared with nonneoplastic mammary tissues in each dietary group (P<0.03), folate status did not significantly affect the extent of DNA methylation. These data suggest that dietary folate deficiency of a moderate degree suppresses, whereas folate supplementation at 4x the basal dietary requirement does not significantly modulate, mammary tumorigenesis in this model. The role of folate in mammaray tumorigenesis needs to be clarified for safe and effective prevention of breast cancer.

## INTRODUCTION

Breast cancer is the most common cancer and the second leading cause of cancer deaths in women in the United States (1). Genetic predisposition (2-4) and hormonal/reproductive factors (5-7), two important determinants of breast cancer risk, are not readily modifiable. Therefore, much effort has been directed towards identifying potentially modifiable dietary and lifestyle factors that would lead to the prevention of breast cancer. Although epidemiological and animal studies have suggested that dietary factors, such as fat, fiber, vegetables and fruits, antioxidants and alcohol, may influence breast cancer risk, effects of these factors on breast cancer risk are inconsistent and contradictory (1). As such, prevention of breast cancer through dietary modifications remains an elusive and challenging task.

Folate, a water-soluble B-vitamin and important co-factor in one-carbon metabolism, has recently been identified as an important nutritional factor that may modulate carcinogenesis (8-10). The role of folate in carcinogenesis has been best studied for colorectal cancer (8-10). The majority of over 25 published epidemiological studies indicate that dietary folate intake and blood folate levels are inversely associated with colorectal cancer risk (8-10). Collectively, these studies suggest an ~40% reduction in the risk of colorectal neoplasms in subjects with highest dietary folate intake compared with those with the lowest intake (8-10). These studies also suggest that a modest reduction in folate status is sufficient to enhance colorectal cancer risk (8-10). Animal studies have also been generally supportive of a causal relationship between folate depletion and colorectal cancer risk as well as a dose-dependent protective effect of modest levels of dietary folate supplementation (4-10X) above the basal dietary requirement on the development and progression of colorectal neoplasms (11-15). Animal studies have also shown that the dose and timing of folate intervention are critical in providing safe and effective

chemoprevention; exceptionally high supplemental folate levels (12, 16, 17) and folate intervention after microscopic neoplastic foci are established in the colorectal mucosa (13, 14) promote, rather than suppress, colorectal carcinogenesis. An accumulating body of evidence suggests that folate status may also play a modulatory role in the development of several other malignancies (e.g. lung, pancreas, stomach, cervix, esophagus, brain and leukemia) (8-10). The precise nature and magnitude of the relationship between folate status and the risk of these malignancies, however, are less clearly defined compared with colorectal cancer.

The relationship between folate status and breast cancer risk has just begun to be reported in the epidemiological literature. Among dietary factors implicated in the development of breast cancer, the inverse relationship between the consumption of vegetables and fruits (the major source of dietary folate) and breast cancer risk (18) and the positive correlation between the intake of alcohol (folate antagonist) and breast cancer risk (19, 20) have been most consistent (1). Among 9 published case-control studies that investigated the relationship between dietary folate intake and breast cancer risk, 7 showed either a significant or equivocal inverse relationship that was not statistically significant, that became nonsignificant after adjustment, or that could not be distinguished from other factors in their relation to risk (21-27), whereas 2 showed an unequivocal null association (28, 29). In some studies, the observed inverse association was further modified by the intake of alcohol and other folate cofactors (e.g. methionine, vitamins $B_6$ and $B_{12}$) (24, 26, 27). One nested case-control study, using stored serum samples, found no association between serum folate and breast cancer risk (30). Two large prospective studies have shown a weak inverse association between the total or dietary intake of folate and breast cancer risk (31, 32). These prospective studies, however, have indicated that low intakes of folate increase, whereas high intakes of folate decrease, breast cancer risk among

women who regularly consume alcohol (31, 32), supporting folate-alcohol interactions in breast carcinogenesis observed in case-control studies (24, 26, 27).

Two animal studies published to date have produced conflicting results concerning the effect of folate on mammary tumorigenesis. In mice with confirmed spontaneous mammary cancers, daily intravenous injections of fermentation *Lactobacillus casei* factor (pteroyltriglutamate) significantly regressed mammary tumors and decreased new mammary tumor formation and lung metastases (33). Another study employing the *N*-methyl-*N*-nitrosourea (MNU) rat model showed that a folate-deficient diet provided during the initiation phase of mammary tumorigenesis significantly reduced tumor multiplicity and increased tumor latency compared with a control and folate-supplemented diet (34). The incidence of mammary tumors, however, was not significantly different among these groups (34). Several inherent limitations associated with these animal studies, however, preclude a definitive conclusion concerning the effect of folate on mammary tumorigenesis.

Because only few modifiable risk factors for breast cancer exist, recent epidemiological observations which suggest that folate deficiency increases, whereas supplementation reduces, breast cancer risk merit further consideration. Folate is an ideal agent for potential chemoprevention of breast cancer. It is a natural vitamin, inexpensive, virtually free of side effects (35), and possesses biologically plausible mechanisms for cancer prevention (8-10). However, the results from published epidemiological and animal studies have been neither consistent nor convincing. Furthermore, a growing body of evidence suggests that folate possesses the dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention (11-17). Folate deficiency has an inhibitory, whereas folate supplementation has a promoting, effect on progression of established neoplasms (11-17). By contrast, folate

deficiency in normal epithelial tissues appears to predispose them to neoplastic transformation, and modest levels of folate supplementation suppress the development of tumors in normal tissues (11-17). Therefore, the potential effect of folate chemoprevention needs to be clearly elucidated in appropriate animal models before folate supplementation can be considered in humans. Given these considerations, this study investigated the effects of dietary folate deficiency and supplementation on the development and progression of mammary tumors in the well-established MNU rat model of breast cancer. Notwithstanding the limitations associated with animal models, the MNU rat model is widely used to determine the effects of dietary factors on mammary tumorigenesis for the following reasons: (a) histological similarities of adenocarcinomas to human breast cancer; (b) local invasiveness and metastatic potential; (c) a clear operational distinction between the initiation and promotion stages; and (d) hormonally dependent mammary tumorigenesis (36-40). Given the role of folate in DNA methylation, an important epigenetic determinant in carcinogenesis (41, 42), we also investigated whether dietary folate modulates genomic DNA methylation in MNU-induced mammary tumorigenesis.

## METHODS

This study was approved by the Animal Care Committee of the University of Toronto.

### Animals and Dietary Intervention

Pathogen-free, weanling female Sprague-Dawley rats (60-75 g; Charles River Laboratories, St. Constant, Quebec, Canada) were randomly assigned to receive an amino acid-defined diet (Dyets, Bethlehem, PA) (43) containing either 0 (n=22), 2 (n=20) or 8 (n=20) mg folic acid/kg diet from weaning at 3 weeks of age for 27 weeks through the MNU treatment (at 50 days of age). Rats were singly housed and maintained at 24 ± 2°C at 50% humidity with a 12 h light/dark cycle. This strain of rats was chosen due to its increased sensitivity to chemically induced mammary tumorigenesis in comparison to Fisher rats, which are of an intermediate sensitivity (44). These diets constitute a standard method of inducing folate deficiency or providing supplemental dietary folate in rodents (43) and have been utilized extensively in previous studies of folate and colorectal cancer (11-14). The diet containing 0 mg folic acid/kg produces progressive folate deficiency of a moderate degree without anemia, growth retardation or premature death through weeks 3-5, after which systemic folate indicators stabilize (11). Although this diet is completely devoid of folate, severe folate deficiency is not induced because of de novo synthesis of folate by intestinal bacteria, some of which is incorporated into the tissue folate of the host (45). Succinylsulphathiazole, which is conventionally used to create a severe folate deficiency (46-48), was not used in this study for the following reasons: (a) we wanted to avoid severe folate deficiency, which predictably causes severe growth retardation and premature death beyond 5-6 weeks (46-48); (b) the consensus of epidemiological (8-10) and animal (11-14) studies indicate that moderate depletion of folate is sufficient to enhance carcinogenesis. This folate-deficient diet is identical to that associated with an increased risk of colorectal neoplasms

in previous animal studies using a chemical colorectal carcinogen or genetically engineered murine models of colorectal cancer (11-14). Two mg folic acid/kg diet is generally accepted as the basal dietary requirement for rodents (49). The diet containing 8 mg folic acid/kg represents folate supplementation 4x the basal dietary requirement. This level of folate was chosen because the 8 mg/kg level has consistently provided a degree of chemoprevention against colorectal cancer in previous rodent studies (11-13).

These diets contained 50 g cellulose/kg, 60% of the calories as carbohydrates, 23% as fat (or 10% by weight), and 17% as L-amino acids (43). The amount of methyl donors, methionine, choline and vitamin $B_{12}$, 8.2 g, 2.0 g and 50 $\mu$g per kg diet, respectively. The detailed composition of the diets has been published previously (14, 43). Diets and water were provided ad libitum.

**MNU Administration**

At 50 days of age, all rats received one intraperitoneal injection of MNU (50 mg/kg body weight; Sigma Chemical Co., St. Louis, MO). A single injection of 50 mg MNU/kg has become the standard dosage due to its rapid induction and high incidence of mammary tumors combined with minimal toxicity and short latency period of 3-6 months (36, 37). The intraperitoneal injection of MNU is considered to be superior to the intravenous or subcutaneous routes in producing mammary tumors as it has been shown to be a simple, rapid, reliable, reproducible and less variable method of MNU administration (38).

**Observation Parameters**

Body weights were recorded weekly. The daily food consumption of each group was measured on a predetermined day of each week. All rats were palpated for mammary tumors once a week beginning 4 weeks after MNU administration. The number, size and location of

each tumor were recorded in a manner that, after histological diagnosis, the time of appearance of the cancers could be determined. All the rats were monitored daily for clinical evidence of illness or morbidity and those approaching a moribund state were promptly euthanized. In addition, rats with tumor burden exceeding 10% of body weight, tumors >15-20 mm in diameter, tumors that impaired normal movement of the animals, and ulcerating tumors were immediately euthanized during the study.

**Sample Collection and Analysis of Mammary Tumors**

Using a preheparinized needle blood was withdrawn from the tail of each rat within a week of MNU injection and from the heart at necropsy into Vacutaner tubes containing EDTA and centrifuged at 800 X g for 10 min at 4°C. Serum was stored at -70°C in 0.5% ascorbic acid for the serum folate assay. Given the latency period of 3-6 months associated with a single intraperitoneal MNU injection and the average duration for the systemic and tissue folate levels to stabilize, the rats were sacrificed by carbon dioxide inhalation followed by cervical dislocation at 23 weeks after MNU injection (27 weeks after dietary intervention or 30 weeks of age). The liver from each rat was harvested, snap-frozen and stored at -70°C for hepatic folate concentration determination. All macroscopic mammary tumors were counted, excised and weighed, and diameters of each tumor were measured using a digital caliper for final tumor volume computation in a blinded fashion. One-half of each macroscopic tumor was snap-frozen in liquid nitrogen and stored at -70°C for DNA extraction. The other half of the tumor was fixed in 10% neutral buffered formalin, processed in a standard manner for hematoxylin-eosin (H&E) staining, and histologically analyzed according to Russo et al. (39) by three experienced pathologists (R.R., C.M., and A.M.) blinded to the study group independently. In the case of a discrepancy, two similar interpretations were utilized for the final analysis. Normal mammary

tissue was also excised at necropsy from each rat, snap-frozen in liquid nitrogen and stored at -70°C for DNA extraction and mammary tissue folate determination.

**Folate Concentration Determination**

Serum folate concentrations were determined by a standard microbiological microtiter plate assay using *Lactobacillus casei* (50). Hepatic and normal mammary tissue folate concentrations were measured by the same microbiologic assay (50), utilizing a previously described method for the determination of tissue folates (51).

**DNA Extraction**

DNA from normal mammary tissues and mammary tumors was extracted by a standard technique using a lysis buffer containing proteinase K followed by phenol, chloroform, and isoamyl alcohol organic extraction (52). The size of DNA estimated by agarose-gel electrophoresis was >20 kb in all instances. No RNA contamination was detected on agarose-gel electrophoresis. The final preparations had a ratio of $A_{260}$ to $A_{280}$ between 1.8 and 2.0. The concentration of each DNA sample was determined as the mean of 3 independent spectrophotometric readings.

**Genomic DNA Methylation Determination**

The methylation status of cytosine-guanine (CpG) sites in genomic DNA from normal mammary tissues and mammary tumors was determined by the in vitro methyl acceptance capacity of DNA using [$^3$H-methyl]S-adenosylmethionine (SAM) as a methyl donor and a prokaryotic CpG DNA methyltransferase, Sss1, as previously described (12, 14, 47, 53). The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous [$^3$H-methyl] incorporation. Briefly, mammary tumor and non-neoplastic mammary gland DNA (500 ng) was incubated with 2.0 $\mu$Ci

of [$^3$H-methyl]SAM (New England Nuclear, Boston, MA), 3 units of Sss1 methylase (New England Biolabs, Beverly, MA), and 1X Sss1 methylation buffer [120mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM EDTA, 1 mM dithiothreitol] in a total volume of 30 $\mu$L for 1 hour at 30°C. The Sss1 was inactivated by incubating at 65°C for 10 minutes. The in vitro methylated DNA was isolated from a 15 $\mu$l aliquot of the reaction mixture by filtration on a Whatman DE-81 ion-exchange filter (Fisher Scientific, Springfield, NJ). The DNA was washed three times with 0.5 M sodium phosphate buffer (pH 7.0), air-dried and the radioactivity of the DNA retained in the filters was measured by scintillation counting using a nonaqueous scintillation fluor. The amount of radiolabel bound to a filter from an incubation mixture without DNA (control) was used as background and was subtracted from the values obtained with mixtures containing DNA. The background value was always <1% of the uptake observed with DNA samples. All analyses were performed in duplicate.

**Statistical Analysis**

Between-group comparisons of continuous variables were assessed using the Kruskal-Wallis and Mann-Whitney non-parameteric tests. For categorical response variables, differences among the groups were assessed by Pearson chi-square test. Differences in genomic DNA methylation between normal mammary gland and tumor in each diet group was assessed by the Wilcoxon signed ranks test. The Kaplan-Meier survival analysis and the Log Rank test were used to compare the rates of tumor appearance among the three groups. All significance tests were two sided and were considered statistically significant if the observed significance level was <0.05. Results are expressed as mean ± SEM. Statistical analyses were performed using SPSS (version 10).

## RESULTS

### Body Weight and Daily Food Consumption

As shown in Figure 1, growth curves were similar among the three dietary groups; at no time point did the mean body weights differ significantly among the three groups. This finding indicates that folate deficiency in the rats fed 0 mg folate/kg diet was moderate; otherwise, growth retardation or premature death would have occurred (46). The mean daily food consumption, which was determined on a preassigned day of each week, was also similar among the three groups (data not shown).

### Serum, Liver and Normal Mammary Gland Folate Concentrations

At the time of MNU injection (4 weeks of dietary intervention) and at necropsy (27 weeks of dietary intervention), the mean serum folate concentrations were significantly different among the three groups ($P<0.001$; Table 1). The mean serum folate concentrations of the three dietary groups at these two time points were comparable to those observed in rats and mice placed on the corresponding diets for 20-24 weeks in previous studies (11-13, 54). These observations indicate that a sufficient degree of systemic folate deficiency and supplementation was achieved in the folate-deficient and supplemented rats, respectively, at the time of MNU injection and throughout the study period to determine the effect of folate status on MNU-induced mammary tumorigenesis. At necropsy, the hepatic folate concentrations of the three dietary groups were significantly different ($P<0.001$; Table 1), and these levels were comparable with those observed in rats placed on the corresponding diets for 24 weeks in previous studies (11, 55). At necropsy, the mean mammary gland folate concentration of the folate-deficient group was significantly lower than the control and folate-supplemented groups ($P<0.001$) while no significant difference was observed between the control and folate-supplemented groups

(Table 1). This observation suggests that mammary gland folate concentrations reached a plateau beyond the 2 mg folate/kg diet. This finding is probably due to the fact that folate accumulation in tissues is limited by the level of folylpolyglutamate synthetase (FPGS) activity in the setting of substrate excess (56, 57).

**Effects of Dietary Folate on MNU-Induced Mammary Tumorigenesis**

No rats died prematurely or were killed before necropsy in the three dietary groups for reasons other than the presence of large and/or ulcerating tumors as defined in the Methods section. Consistent with previous observations made in the MNU-Sprague-Dawley rat model of mammary tumorigenesis (36-40), >90% of macroscopic mammary tumors in the present study were identified histologically as either adenomas (15%) or adenocarcinomas (85%). There was an excellent agreement in histological diagnosis of either adenoma or adenocarcimona among the three study pathologists (kappa statistic = 0.95). The analyses pertaining to mammary tumors were performed for the combination of adenocarcinomas and adenomas and for adenocarcinomas alone. There were not a sufficient number of adenomas for independent analysis.

As shown in Figure 2A, there was a trend towards a significant difference in the rates of appearance of either adenocarcinomas or adenomas among the three dietary groups (P=0.07). This was mainly due to the difference between the folate-deficient and control groups (P=0.02). By contrast, there was no significant difference between the folate-deficient and supplemented groups (P=0.11), and between the control and folate-supplemented groups (P=0.72). We excluded one outlier from the folate-deficient group, which harbored a total of 9 adenocarcinomas and adenomas, and this strengthened the overall comparison (P=0.06). When the analysis was confined to adenocarcinomas alone, similar patterns were observed. There was a

trend towards a significant difference in the rates of appearance of adenocarcinomas among the three groups (P-overall=0.08; P=0.05 between the folate-deficient and control groups; P=0.04 between the folate-deficient and supplemented groups; P=0.83 between the control and folate-supplemented groups; Figure 2B).

There was a trend towards a significant difference in the incidence of histologically confirmed adenocarcinomas and adenomas at necropsy (P=0.057; Table 2). This was mainly due to the difference between the folate-deficient and control groups (P=0.02); there was no significant difference between the folate-deficient and supplemented groups (P=0.19) or between the control and folate-supplemented groups (P=0.20). When the outlier was excluded from the folate-deficient group, the overall difference in the incidence of adenocarcinomas and adenomas became significant (P=0.043) with a similar trend in between-group comparisons. As shown in Table 2, there was no significant difference in mean tumor latency (mean time to appearance of first palpable tumor), multiplicity (mean number of tumors per tumor-bearing rat), volume or weight among the three groups, whether or not the outlier was included or excluded in the analyses. When the analyses were confined to adenocarcinomas alone, no significant difference in the incidence and mean tumor latency, multiplicity, volume or weight among the three groups was observed (Table 3).

**Genomic DNA Methylation Status**

As shown in Figure 3, the degree of $^{3}$H-methyl incorporation into DNA of the mammary adenocarcinoma and into DNA from the pair-matched non-neoplastic mammary tissue was not significantly different among the three dietary groups. However, the degree of $^{3}$H-methyl incorporation into DNA of the mammary adenocarcinomas, which is inversely related to the extent of genomic DNA methylation, was 4- to 5-fold higher than that of non-neoplastic

mammary tissue within each dietary group ($P<0.03$; Figure 3), indicating a significantly lower degree of genomic DNA methylation in the adenocarcinomas compared with the normal mammary tissue.

## DISCUSSION

Our data suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats. By contrast, dietary folate supplementation at 4x the basal dietary requirement does not appear to modulate mammary tumorigenesis in this model. These observations contradict the generally accepted notion based on epidemiologic evidence, which suggests that folate deficiency enhances, whereas folate supplementation suppresses, the development of breast cancer (21-27, 31, 32). Epidemiologic evidence available thus far has not been consistent nor has it provided unequivocal support for the purported inverse relationship between folate status and breast cancer risk (21-32). However, none of the published epidemiologic studies has demonstrated a positive association between folate status and breast cancer risk. Some epidemiological studies have suggested that folate status alone may not be sufficient in modifying breast cancer risk. However, with alcohol consumption folate deficiency potentiates, whereas folate supplementation reduces, the risk of breast caner (24, 26, 27, 31, 32). Furthermore, some sudies have suggested that folate status may modify breast cancer risk in conjunction with other dietary factors involved in one-carbon metabolism such as methionine, vitamins $B_6$ and $B_{12}$ (27, 31). A recent study has suggested that the modulating effect of folate status on breast cancer risk is further modified by certian genotypes (58).

Our data differ from the promoting and protective effect of folate deficiency and supplementation, respectively, on intestinal tumorigenesis observed in the chemical carcinogen (dimethylhydrazine) and genetically engineered rodent models utilizing the same diets employed in the present study (11-14, 54). However, some animal studies have suggested that folate status may have the opposite effect on intestinal tumorigenesis depending on the timing and dose of folate intervention (12-14, 16, 17). The contradicting effect of dietary folate on mammary and

intestinal tumorigenesis in animal models using the same diets suggests that folate may modulate carcinogenesis in a tissue- and/or carcinogen-specific manner. The results from the present study are, however, consistent with those of a previous study that investigated the effect of dietary folate deficiency and supplementation on initiation and early promotion of MNU-induced mammary tumorigenesis in Fischer 344 rats (34). Baggott and colleagues performed a study in which rats were fed a casein-based AIN-76A diet containing either 0, 2 or 40 mg folic acid/kg diet, or 20 mg folinic acid/kg diet at weaning (27 days of age) for 30 days, injected with MNU intravenously (50 mg/kg body weight), and subsequently fed the control diet containing 2 mg folic acid/kg for 180 days. Glycine and succinylsulfathiazole (10 g/kg diet) were added to the diet to potentiate folate deficiency. Plasma folate concentrations were 15 ± 5, 77 ±15 and 218± 47 ng/mL for the 0, 2 and 40 mg folic acid/kg diet groups at the time of MNU injection and 79 ± 8, 58 ± 6 and 56 ± 6 ng/mL at necropsy. Although the incidence of mammary cancer was not significantly different among the 4 groups, cancer multiplicity was significantly lower in rats fed the 0 mg folic acid diet than those fed the 2 mg folic acid, the 40 mg folic acid or the 20 mg folinic acid diets; there was no signficant difference in cancer multiplicity among the latter 3 groups. Furthermore, the time required for 50% of the rats to develop palpable mammary cancer was significantly longer in the 0 mg folic acid group than in the 40 mg folic acid or the 20 mg folinic acid group, but was not significantly different from that in the 2 mg folic acid group. Thus, Baggott's study demonstrated that folate deficiency suppressed initiation and early promotion of MNU-induced mammary tumorigenesis (34).

As suggested by Baggott's study (34), the inhibitory effect of folate deficiency on MNU-induced mammary tumorigenesis in rats may be a real effect on initiation and early promotion. Our study suggests that folate deficiency induced throughout the initiation, promotion and

progression phases of MNU-induced mamary tomorigenesis also has an inhibitory effect. However, it is possible that the conventional dose and route of MNU injection employed in the present study might have created an overwhelmingly carcinogenic milieu for folate status to modulate initiation of mammary tumorigenesis. Regardless of the levels of dietary folate, MNU likely induced and established neoplastic foci in mammary tissues. In this setting, folate deficiency likely suppressed the progression of and/or caused regression of established mammary neoplastic foci. This explanation is consistent with the biochemical function of folate. Interruption of folate metabolism in rapidly replicating neoplastic cells to cause ineffective DNA synthesis and hence the inhibition of tumor growth has been the basis of antitumor therapy using antifolate agents (59). It has been shown in experimental models that growth of a transplanted cancer is inhibited in folate-deficient rats (60), that folate deprivation reduces the growth of virally-induced cancers (61), and that the time required for developing nerve sheath tumors in transgenic mice is significantly delayed by restricting the level of folate in the diet (62). Furthermore, previous studies using chemical and genetically-predisposed murine models and the same folate diets as in the present study have demonstrated that folate deficiency suppresses the progression of and induces regression of established intestinal neoplasms (12-14). Therefore, it is possible that the inhibitory effect of folate deficiency on MNU-induced tumorigenesis in this rat model might have been primarily on promotion/progression of established mammary neoplastic foci. The exact stage at which folate deficiency exerts its inhibitory effect on mammary tumorigenesis needs to be clearly defined.

In the present study, dietary folate supplementation at 4x the basal dietary requirement, which has consistently conferred protection against intestinal tumorigenesis in rodents in previous studies (11-14), did not inhibit mammary tumorigenesis when supplemented throughout

the initiation, promotion and progression phases of MNU-induced mammary tumorigenesis. This level of dietary folate supplementation did not promote the progression of MNU-induced mammary neoplastic foci in the present study in contrast to the promoting effect associated with this level of dietary folate supplementation on progression of established intestinal neoplastic foci observed in some studies (13, 14). The lack of effect associated with folate supplementation on mammary tumorigenesis in the present study may be related to the fact that, in spite of significantly higher serum and hepatic folate levels, the mean mammary gland folate concentration of the folate-supplemented rats was not significantly different from that of the controls. Previous studies have demonstrated a dose-responsive tissue saturating effect of folate supplementation above 4x the basal dietary requirement in rat colon (12), and the 8 mg folic acid diet has consistently induced signficantly higher colonic mucosal folate concentrations compared with the 2 mg folic acid (control) diet in rodents (12-14, 48, 55). It is well known that different tissues express different folate requirements and hence different susceptibility to folate deficiency (51). Furthermore, folate accumulation in tissues is limited by the level of FPGS activity in the setting of substrate excess (56, 57). FPGS catalyzes polyglutamation of intracellular folates, thereby allowing the retention of folate that would otherwise be lost because of efflux from the cell (56, 57). Previous studies in animals and in cultured cells have shown that tissue levels of folate reach a plateau when FPGS is saturated from excess folate in the diet or culture medium (12, 56, 57). FPGS activity appears to be the highest in the liver, with lower levels found in other mammalian tissues including the brain, muscle and kidney (57, 63). At present, there is no information in the literature regarding the levels of FPGS activity in normal mammary tissue. It is possible that the levels of FPGS activity in the mammary gland are appreciably lower than the liver or colon and thus tissue folate is saturated at a much lower level

of dietary folate in the mammary gland compared with other tissues. However, it is also possible that higher levels of dietary folate supplementation above 4x the basal dietary requirement may be necessary to increase mammary folate concentrations compared with the control diet. The role of FPGS in mammary gland folate metabolism and the effect of higher folate supplementation above 4x the basal dietary requirement on mammary gland folate concentrations and consequent mammary tumorigenesis need to be clearly elucidated.

One interesting finding in this study is that the extent of genomic DNA methylation is significantly lower in mammary adenocarcinomas than in nonneoplastic mammary tissues regardless of folate status. DNA methylation is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in chromatin modifications and in the development of mutations (41, 42). Neoplastic cells simultaneously harbor widespread genomic DNA hypomethylation and more specific regional areas of hypermethylation (41, 42). Genomic and protooncogene-specific hypomethylation appears to be an early, and consistent, event in carcinogenesis (41, 42). There appears to be a direct correlation between the extent of genomic DNA hypomethylation and tumor progression (41, 42). Genomic hypomethylation results in genomic instability and increased mutations, and protooncogene-specific hypomethylation results in increased gene expression (41, 42). In addition, site-specific hypomethylation at the promoter region of tumor suppressor and mismatch repair genes is an important mechanism in gene silencing in carcinogenesis (41, 42). Although promoter CpG islands hypermethylation of several genes including *BRCA1, ER, p16, E-Cadherin, TMS1, RASSF1*, leading to inactivation of these genes have been observed in human breast cancer (64-69), very few studies have reported genomic hypomethylation in human breast cancer (70, 71). To our knowledge, our study is the first to demonstrate that genomic DNA hypomethylation is an

epigenetic phenomenon associated with MNU-induced mammary tumorigenesis in rats. The extent of genomic DNA methylation in mammary adenocarcinomas and in nonneoplastic mammary tissues was not significantly modulated by folate status. This observation suggests that altered genomic DNA methylation was not a likely mechanism by which folate deficiency suppressed mammary tumorigenesis in our study. Folate, in the form of 5-methyltetrahydrofolate, is involved in remethylation of homocysteine to methionine, which is a precursor of SAM, the primary methyl group donor for most biological methylation reactions (8-10). Although isolated folate deficiency has been shown to induce genomic DNA hypomethylation in circulating lymphocytes in humans (72, 73), this effect has not been demonstrated in the colorectum or liver in rodents fed the same folate-deficient diet employed in the present study (12, 14, 55). However, an extremely severe degree of folate deficiency has been shown to induce genomic (53) and site-specific *p53* (47) DNA hypomethylation in rat liver, although other studies have disputed the finding on genomic DNA methylation (47). Because both site-specific hypo- and hypermethylation play a role in carcinogenesis (41, 42) and because folate may modulate DNA methylation in a site-specific manner (47), it would be of great interest to study site-specific methylation of protooncogenes and tumor suppressor genes implicated in mammary tumorigenesis (2-4) and the effect of folate status.

The strengths of the present study include: (a) the use of the amino acid-defined diet that is widely accepted as the standard means of inducing folate deficiency or providing supplemental dietary folate in rodents; (b) the use of dietary levels of folate that have been shown to modulate the development of other cancers in this strain of rats; (c) measurements of systemic and mammary gland folate concentrations; (d) rigorous histological confirmation of all mammary tumors to ensure an accurate determination of the rate of appearance and other tumor parameters

of adenomas and adenocarcinomas. However, several limitations associated with the present study need to be acknowledged. Firstly, although the dose and route of MNU administration employed in the present study may be appropriate in studies examining the effect of other potential chemopreventive agents in this model, the effect may be too overwhelmingly carcinogenic for folate to modulate. Therefore, the effect observed with dietary folate in the present study may be predominantly on promotion and progression, and not on initiation, of MNU-induced neoplastic foci. Secondly, the fat content of the diets used in the present study was higher than the AIN rodent diets that are more commonly used in experimental mammary tumor studies (10% versus 7% by weight). Animal studies have generally suggested that high fat diets enhance mammary tumorigenesis in rodents (74). Therefore, it is possible that the tumor-promoting effect associated with the higher fat content in our diets might have masked any modulating effect of dietary folate intervention. Thirdly, the mean mammary gland folate concentration associated with folate supplementation was not significantly higher than that of the control diet. Therefore, higher levels of folate supplementation above 4x the basal dietary requirement may be necessary to significantly increase mammary gland folate concentrations and to observe any modulatory effect of folate supplementation on mammary tumorigenesis. Lastly, the number of animals employed in the present study did not allow us to achieve adequate statistical power. It would have required 103 animals in total to be 80% certain of detecting a 35% reduction in tumor incidence associated with folate deficiency compared with the control diet at a 5% level of significance.

In summary, our data suggest that dietary folate deficiency of a moderate degree suppresses MNU-induced mammary tumorigenesis in rats. By contrast, dietary folate supplementation at 4x the basal dietary requirement does not significantly modulate mammary

tumorigenesis. Notwithstanding the limitations associated with this model, our data suggest that the role of folate in mammaray tumorigenesis needs to be clarified in future studies for safe and effective prevention of breast cancer. In light of the findings from the present study, further investigation is warranted to elucidate the role of folate in mammary tumorigenesis. The effect of folate on the initiation and promotion/progression phases of MNU-induced tumorigenesis needs to be clearly defined. Lower doses of MNU, lower fat content and higher levels of folate supplementation may be necessary to clearly elucidate the effect of folate on mammary tumorigenesis in this model. The effect of folate on mammary tumorigenesis observed in the present study needs to be confirmed in other animal models. Given the possible interactions of folate with alcohol and other folate co-factors in modifying breast cancer risk observed in some epidemiological studies (24, 26, 27, 31, 32), these potential interactions merit further investigation.

## ACKNOWLEDGMENTS

We thank the veterinary technologists of the Division of Comparative Medicine, Faculty of Medicine, University of Toronto for the care of the rats used in this study. We also thank Dr. Michael Archer and Ms. Suying Lu for insightful advice and technical assistance.

## REFERENCES


1. World Cancer Research Fund/American Institute for Cancer Research. Food, nutrition and the prevention of cancer: a global perspective. Washington, DC: The American Institute for Cancer Research, 1997.
2. Dillon, D. A., Howe, C. L., Bosari, S., and Costa, J. The molecular biology of breast cancer: accelerating clinical applications. Crit Rev Oncog, *9:* 125-140, 1998.
3. Ellisen, L. W. and Haber, D. A. Hereditary breast cancer. Annu Rev Med, *49:* 425-436, 1998.
4. Martin, A. M. and Weber, B. L. Genetic and hormonal risk factors in breast cancer. J Natl Cancer Inst, *92:* 1126-1135, 2000.
5. Duda, R. B. Risk factors for the development of breast cancer. Compr Ther, *21:* 29-34, 1995.
6. Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. Collaborative Group on Hormonal Factors in Breast Cancer. Lancet, *347:* 1713-1727, 1996.
7. Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer. Lancet, *350:* 1047-1059, 1997.
8. Kim, Y. I. Folate and carcinogenesis: evidence, mechanisms, and implications. J Nutr Biochem, *10:* 66-88, 1999.

9. Kim, Y. I. Folate and cancer prevention: a new medical application of folate beyond hyperhomocysteinemia and neural tube defects. Nutr Rev, *57:* 314-321, 1999.

10. Mason, J. B. and Choi, S. W. Folate and carcinogenesis: developing a unifying hypothesis. Adv Enzyme Regul, *40:* 127-141, 2000.

11. Cravo, M. L., Mason, J. B., Dayal, Y., Hutchinson, M., Smith, D., Selhub, J., and Rosenberg, I. H. Folate deficiency enhances the development of colonic neoplasia in dimethylhydrazine-treated rats. Cancer Res, *52:* 5002-5006, 1992.

12. Kim, Y. I., Salomon, R. N., Graeme-Cook, F., Choi, S. W., Smith, D. E., Dallal, G. E., and Mason, J. B. Dietary folate protects against the development of macroscopic colonic neoplasia in a dose responsive manner in rats. Gut, *39:* 732-740, 1996.

13. Song, J., Medline, A., Mason, J. B., Gallinger, S., and Kim, Y. I. Effects of dietary folate on intestinal tumorigenesis in the $Apc^{Min}$ mouse. Cancer Res, *60:* 5434-5440, 2000.

14. Song, J., Sohn, K. J., Medline, A., Ash, C., Gallinger, S., and Kim, Y. I. Chemopreventive effects of dietary folate on intestinal polyps in *Apc+/-Msh2-/-* mice. Cancer Res, *60:* 3191-3199, 2000.

15. Wargovich, M. J., Jimenez, A., McKee, K., Steele, V. E., Velasco, M., Woods, J., Price, R., Gray, K., and Kelloff, G. J. Efficacy of potential chemopreventive agents on rat colon aberrant crypt formation and progression. Carcinogenesis, *21:* 1149-1155, 2000.

16. Wargovich, M. J., Chen, C. D., Jimenez, A., Steele, V. E., Velasco, M., Stephens, L. C., Price, R., Gray, K., and Kelloff, G. J. Aberrant crypts as a biomarker for colon cancer: evaluation of potential chemopreventive agents in the rat. Cancer Epidemiol Biomarkers Prev, *5:* 355-360, 1996.

17. Le Leu, R. K., Young, G. P., and McIntosh, G. H. Folate deficiency reduces the development of colorectal cancer in rats. Carcinogenesis, *21:* 2261-2265, 2000.

18. Gandini, S., Merzenich, H., Robertson, C., and Boyle, P. Meta-analysis of studies on breast cancer risk and diet: the role of fruit and vegetable consumption and the intake of associated micronutrients. Eur J Cancer, *36:* 636-646, 2000.

19. Smith-Warner, S. A., Spiegelman, D., Yaun, S. S., van den Brandt, P. A., Folsom, A. R., Goldbohm, R. A., Graham, S., Holmberg, L., Howe, G. R., Marshall, J. R., Miller, A. B., Potter, J. D., Speizer, F. E., Willett, W. C., Wolk, A., and Hunter, D. J. Alcohol and breast cancer in women: a pooled analysis of cohort studies. JAMA, *279:* 535-540, 1998.

20. Bagnardi, V., Blangiardo, M., Vecchia, C. L., and Corrao, G. A meta-analysis of alcohol drinking and cancer risk. Br J Cancer, *85:* 1700-1705, 2001.

21. Graham, S., Hellmann, R., Marshall, J., Freudenheim, J., Vena, J., Swanson, M., Zielezny, M., Nemoto, T., Stubbe, N., and Raimondo, T. Nutritional epidemiology of postmenopausal breast cancer in western New York. Am J Epidemiol, *134:* 552-566, 1991.

22. Freudenheim, J. L., Marshall, J. R., Vena, J. E., Laughlin, R., Brasure, J. R., Swanson, M. K., Nemoto, T., and Graham, S. Premenopausal breast cancer risk and intake of vegetables, fruits, and related nutrients. J Natl Cancer Inst, *88:* 340-348, 1996.

23. Ronco, A., De Stefani, E., Boffetta, P., Deneo-Pellegrini, H., Mendilaharsu, M., and Leborgne, F. Vegetables, fruits, and related nutrients and risk of breast cancer: a case-control study in Uruguay. Nutr Cancer, *35:* 111-119, 1999.

24. Rohan, T. E., Jain, M. G., Howe, G. R., and Miller, A. B. Dietary folate consumption and breast cancer risk. J Natl Cancer Inst, *92:* 266-269, 2000.

25. Levi, F., Pasche, C., Lucchini, F., and La Vecchia, C. Dietary intake of selected micronutrients and breast-cancer risk. Int J Cancer, *91:* 260-263, 2001.

26. Negri, E., La Vecchia, C., and Franceschi, S. Re: dietary folate consumption and breast cancer risk. J Natl Cancer Inst, *92:* 1270-1271, 2000.

27. Shrubsole, M. J., Jin, F., Dai, Q., Shu, X. O., Potter, J. D., Hebert, J. R., Gao, Y. T., and Zheng, W. Dietary folate intake and breast cancer risk: results from the Shanghai Breast Cancer Study. Cancer Res, *61:* 7136-7141, 2001.

28. Thorand, B., Kohlmeier, L., Simonsen, N., Croghan, C., and Thamm, M. Intake of fruits, vegetables, folic acid and related nutrients and risk of breast cancer in postmenopausal women. Public Health Nutr, *1:* 147-156, 1998.

29. Potischman, N., Swanson, C. A., Coates, R. J., Gammon, M. D., Brogan, D. R., Curtin, J., and Brinton, L. A. Intake of food groups and associated micronutrients in relation to risk of early-stage breast cancer. Int J Cancer, *82:* 315-321, 1999.

30. Wu, K., Helzlsouer, K. J., Comstock, G. W., Hoffman, S. C., Nadeau, M. R., and Selhub, J. A prospective study on folate, B12, and pyridoxal 5'-phosphate (B6) and breast cancer. Cancer Epidemiol Biomarkers Prev, *8:* 209-217, 1999.

31. Zhang, S., Hunter, D. J., Hankinson, S. E., Giovannucci, E. L., Rosner, B. A., Colditz, G. A., Speizer, F. E., and Willett, W. C. A prospective study of folate intake and the risk of breast cancer. JAMA, *281:* 1632-1637, 1999.

32. Sellers, T. A., Kushi, L. H., Cerhan, J. R., Vierkant, R. A., Gapstur, S. M., Vachon, C. M., Olson, J. E., Therneau, T. M., and Folsom, A. R. Dietary folate intake, alcohol, and risk of breast cancer in a prospective study of postmenopausal women. Epidemiology, *12:* 420-428, 2001.

33. Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., and keresztesy, J. C. The influence of liver L. casei factor on spontaneous breast cancer in mice. Science, *104:* 436-437, 1946.

34. Baggott, J. E., Vaughn, W. H., Juliana, M. M., Eto, I., Krumdieck, C. L., and Grubbs, C. J. Effects of folate deficiency and supplementation on methylnitrosourea-induced rat mammary tumors. J Natl Cancer Inst, *84:* 1740-1744, 1992.

35. Campbell, N. R. How safe are folic acid supplements? Arch Intern Med, *156:* 1638-1644, 1996.

36. Rose, D. P., Pruitt, B., Stauber, P., Erturk, E., and Bryan, G. T. Influence of dosage schedule on the biological characteristics of N-nitrosomethylurea-induced rat mammary tumors. Cancer Res, *40:* 235-239, 1980.

37. McCormick, D. L., Adamowski, C. B., Fiks, A., and Moon, R. C. Lifetime dose-response relationships for mammary tumor induction by a single administration of N-methyl-N-nitrosourea. Cancer Res, *41:* 1690-1694, 1981.

38. Thompson, H. J. and Adlakha, H. Dose-responsive induction of mammary gland carcinomas by the intraperitoneal injection of 1-methyl-1-nitrosourea. Cancer Res, *51:* 3411-3415, 1991.

39. Russo, J., Russo, I. H., Rogers, A. E., Van Zwienten, M. J., and Gusterson, B. Tumors of mammary gland. *In:* V. Turusov and U. Mohr (eds.), Pathology of tumours in laboratory animals, Vol. 1, pp. 47-78. Lyon: IARC Scientific Publications, 1990.

40. Russo, I. H. and Russo, J. Mammary gland neoplasia in long-term rodent studies. Environ Health Perspect, *104:* 938-967, 1996.

41. Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M., and Issa, J. P. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res, *72:* 141-196, 1998.

42. Jones, P. A. and Laird, P. W. Cancer epigenetics comes of age. Nat Genet, *21:* 163-167, 1999.

43. Walzem, R. L. and Clifford, A. J. Folate deficiency in rats fed diets containing free amino acids or intact proteins. J Nutr, *118:* 1089-1096, 1988.

44. Chan, P. C., Head, J. F., Cohen, L. A., and Wynder, E. L. Influence of dietary fat on the induction of mammary tumors by N-nitrosomethylurea: associated hormone changes and differences between Sprague-Dawley and F344 rats. J Natl Cancer Inst, *59:* 1279-1283, 1977.

45. Rong, N., Selhub, J., Goldin, B. R., and Rosenberg, I. H. Bacterially synthesized folate in rat large intestine is incorporated into host tissue folyl polyglutamates. J Nutr, *121:* 1955-1959, 1991.

46. Clifford, A. J., Wilson, D. S., and Bills, N. D. Repletion of folate-depleted rats with an amino acid-based diet supplemented with folic acid. J Nutr, *119:* 1956-1961, 1989.

47. Kim, Y. I., Pogribny, I. P., Basnakian, A. G., Miller, J. W., Selhub, J., James, S. J., and Mason, J. B. Folate deficiency in rats induces DNA strand breaks and hypomethylation within the p53 tumor suppressor gene. Am J Clin Nutr, *65:* 46-52, 1997.

48. Kim, Y. I., Shirwadkar, S., Choi, S. W., Puchyr, M., Wang, Y., and Mason, J. B. Effects of dietary folate on DNA strand breaks within mutation-prone exons of the p53 gene in rat colon. Gastroenterology, *119:* 151-161, 2000.

49. Reeves, P. G., Nielsen, F. H., and Fahey, G. C., Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr, *123:* 1939-1951, 1993.

50. Tamura, T. Microbiological assay of folate. *In:* M. F. Picciano, E. L. R. Stockstad, and J. F. Gregory (eds.), Folic acid metabolism in health and disease, pp. 121-137. New York: Wiley-Liss, 1992.

51. Varela-Moreiras, G. and Selhub, J. Long-term folate deficiency alters folate content and distribution differentially in rat tissues. J Nutr, *122:* 986-991, 1992.

52. Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R., and Berns, A. Simplified mammalian DNA isolation procedure. Nucleic Acids Res, *19:* 4293, 1991.

53. Balaghi, M. and Wagner, C. DNA methylation in folate deficiency: use of CpG methylase. Biochem Biophys Res Commun, *193:* 1184-1190, 1993.

54. Carrier, J., Medline, A., Sohn, K. J., Hwang, S., and Kim, Y. I. Effects of dietary folate on colorectal carcinogenesis in a genetically-predisposed murine model of ulcerative colitis-associated colon cancer. Gastroenterology, *120:* A447, 2001.

55. Kim, Y. I., Christman, J. K., Fleet, J. C., Cravo, M. L., Salomon, R. N., Smith, D., Ordovas, J., Selhub, J., and Mason, J. B. Moderate folate deficiency does not cause global hypomethylation of hepatic and colonic DNA or c-myc-specific hypomethylation of colonic DNA in rats. Am J Clin Nutr, *61:* 1083-1090, 1995.

56. Lowe, K. E., Osborne, C. B., Lin, B. F., Kim, J. S., Hsu, J. C., and Shane, B. Regulation of folate and one-carbon metabolism in mammalian cells. II. Effect of folylpoly-gamma-glutamate synthetase substrate specificity and level on folate metabolism and folylpoly-

gamma-glutamate specificity of metabolic cycles of one-carbon metabolism. J Biol Chem, *268:* 21665-21673, 1993.

57. Shane, B. Folate chemistry and metabolism. *In:* L. B. Bailey (ed.), Folate in health and disease, pp. 1-22. New York: Marcel Dekker, 1995.

58. Goodman, J. E., Lavigne, J. A., Wu, K., Helzlsouer, K. J., Strickland, P. T., Selhub, J., and Yager, J. D. COMT genotype, micronutrients in the folate metabolic pathway and breast cancer risk. Carcinogenesis, *22:* 1661-1665, 2001.

59. Kamen, B. Folate and antifolate pharmacology. Semin Oncol, *24:* S18-30-S18-39, 1997.

60. Rosen, F. and Nichol, C. A. Inhibition of the growth of an amethopterin-refractory tumor by dietary restriction of folic acid. Cancer Res, *22:* 495-500, 1962.

61. Little, P. A., Sampath, A., and Paganelli, V. The effect of folic acid and its antagonists on Rous chicken sarcoma. Trans NY Acad Sci Series II, *10:* 91-98, 1948.

62. Bills, N. D., Hinrichs, S. H., Morgan, R., and Clifford, A. J. Delayed tumor onset in transgenic mice fed a low-folate diet. J Natl Cancer Inst, *84:* 332-337, 1992.

63. Synold, T. W., Willits, E. M., and Barredo, J. C. Role of folylpolygutamate synthetase (FPGS) in antifolate chemotherapy; a biochemical and clinical update. Leuk Lymphoma, *21:* 9-15, 1996.

64. Ottaviano, Y. L., Issa, J. P., Parl, F. F., Smith, H. S., Baylin, S. B., and Davidson, N. E. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res, *54:* 2552-2555, 1994.

65. Rice, J. C., Massey-Brown, K. S., and Futscher, B. W. Aberrant methylation of the BRCA1 CpG island promoter is associated with decreased BRCA1 mRNA in sporadic breast cancer cells. Oncogene, *17:* 1807-1812, 1998.

66. Graff, J. R., Herman, J. G., Lapidus, R. G., Chopra, H., Xu, R., Jarrard, D. F., Isaacs, W. B., Pitha, P. M., Davidson, N. E., and Baylin, S. B. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res, *55:* 5195-5199, 1995.

67. Foster, S. A., Wong, D. J., Barrett, M. T., and Galloway, D. A. Inactivation of p16 in human mammary epithelial cells by CpG island methylation. Mol Cell Biol, *18:* 1793-1801, 1998.

68. Burbee, D. G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., Sekido, Y., Latif, F., Milchgrub, S., Toyooka, S., Gazdar, A. F., Lerman, M. I., Zabarovsky, E., White, M., and Minna, J. D. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J Natl Cancer Inst, *93:* 691-699, 2001.

69. Conway, K. E., McConnell, B. B., Bowring, C. E., Donald, C. D., Warren, S. T., and Vertino, P. M. TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. Cancer Res, *60:* 6236-6242, 2000.

70. Soares, J., Pinto, A. E., Cunha, C. V., Andre, S., Barao, I., Sousa, J. M., and Cravo, M. Global DNA hypomethylation in breast carcinoma: correlation with prognostic factors and tumor progression. Cancer, *85:* 112-118, 1999.

71. Bernardino, J., Roux, C., Almeida, A., Vogt, N., Gibaud, A., Gerbault-Seureau, M., Magdelenat, H., Bourgeois, C. A., Malfoy, B., and Dutrillaux, B. DNA hypomethylation in breast cancer: an independent parameter of tumor progression? Cancer Genet Cytogenet, *97:* 83-89, 1997.

72. Rampersaud, G. C., Kauwell, G. P., Hutson, A. D., Cerda, J. J., and Bailey, L. B. Genomic DNA methylation decreases in response to moderate folate depletion in elderly women. Am J Clin Nutr, *72:* 998-1003, 2000.

73. Jacob, R. A., Gretz, D. M., Taylor, P. C., James, S. J., Pogribny, I. P., Miller, B. J., Henning, S. M., and Swendseid, M. E. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. J Nutr, *128:* 1204-1212, 1998.

74. Cohen, L. A., Thompson, D. O., Maeura, Y., Choi, K., Blank, M. E., and Rose, D. P. Dietary fat and mammary cancer. I. Promoting effects of different dietary fats on N-nitrosomethylurea-induced rat mammary tumorigenesis. J Natl Cancer Inst, *77:* 33-42, 1986.

## FIGURE LEGENDS

**Figure 1:** Mean body weights of rats fed amino acid-defined diets containing either 0 (deficient), 2 (control, daily basal dietary requirement) or 8 (supplemented) mg folic acid/kg diet starting at 3 weeks of age. Values are mean ± SEM.

**Figure 2A:** The rate of appearance of either mammary adenomas or adenocarcinomas among the three dietary groups (P-overall=0.07; P=0.02 between the 0 and 2 mg folic acid groups; P=0.11 between the 0 and 8 mg folic acid groups; P=0.72 between the 2 and 8 mg folic acid groups by the Kaplan-Meier survival analysis and Log Rank test). Excluding 1 outlier in the 0 mg folic acid group, which harbored a total of 9 adenocarcinomas and adenomas, strengthened the overall comparison (P=0.06) with similar patterns in between-groups comparisons (P=0.02 between the 0 and 2 mg folic acid groups; P=0.09 between the 0 and 8 mg folic acid groups; P=0.72 between the 2 and 8 mg folic acid groups).

**Figure 2B:** The rate of appearance of mammary adenocarcinomas among the three dietary groups (P-overall=0.08; P=0.05 between the 0 and 2 mg folic acid groups; P=0.04 between the 0 and 8 mg folic acid groups; P=0.83 between the 2 and 8 mg folic acid groups by the Kaplan-Meier survival analysis and Log Rank test).

**Figure 3:** Effects of dietary folate on genomic DNA methylation in mammary adenocarcinomas and non-neoplastic mammary tissues as determined by the in vitro methyl acceptance assay. The manner in which this assay is performed produces a reciprocal relationship between the endogenous DNA methylation status and the exogenous [$^3$H-methyl] incorporation into DNA.

Different letters within each dietary group denote significant differences by the Wilcoxon signed ranks test at $P<0.03$). Values are mean ± SEM.

Table 1: Serum, hepatic and mammary gland folate concentrations[1]

| | At the time of MNU injection (4 weeks of dietary intervention) | | | At necropsy (27 weeks of dietary intervention) | | |
|---|---|---|---|---|---|---|
| **Diet (mg folate/kg diet)** | 0 | 2 | 8 | 0 | 2 | 8 |
| **Serum folate (ng/ml)** | $13.75\pm1.04^{a}$ | $68.82\pm2.65^{b}$ | $100.78\pm2.82^{c}$ | $10.53\pm1.16^{a}$ | $49.24\pm3.16^{b}$ | $77.35\pm2.33^{c}$ |
| **Hepatic folate (μg/g tissue)** | | | | $2.18\pm0.32^{a}$ | $6.42\pm0.35^{b}$ | $9.01\pm0.37^{c}$ |
| **Mammary folate (ng/g tissue)** | | | | $56.20\pm7.54^{a}$ | $178.13\pm29.06^{b}$ | $175.94\pm24.32^{b}$ |

1. Results are expressed as mean ± SEM. Means in a row with different letters at each time point significantly differ at $P<0.001$.

**Table 2: Effects of dietary folate on the incidence, latency, multiplicity, volume and weight of mammary adenocarcinomas and adenomas[1]**

| Diet (mg folate/kg diet) | 0 | 2 | 8 | P-value |
|---|---|---|---|---|
| Incidence (%) | 57[a] | 90[b] | 75[a,b] | 0.057 |
| Incidence (%)* | 55[a] | 90[b] | 75[a,b] | 0.043 |
| Mean latency (weeks post-MNU injection) | 17.83±1.35 | 17.06±1.11 | 15.00±1.36 | 0.29 |
| Mean multiplicity | 3.67±1.03 | 2.87±0.53 | 2.20±0.34 | 0.72 |
| Mean volme ($cm^3$) | 2.83±0.80 | 2.88±0.58 | 1.49±0.39 | 0.28 |
| Mean weight (g) | 0.86±0.23 | 1.03±0.23 | 0.58±0.14 | 0.45 |

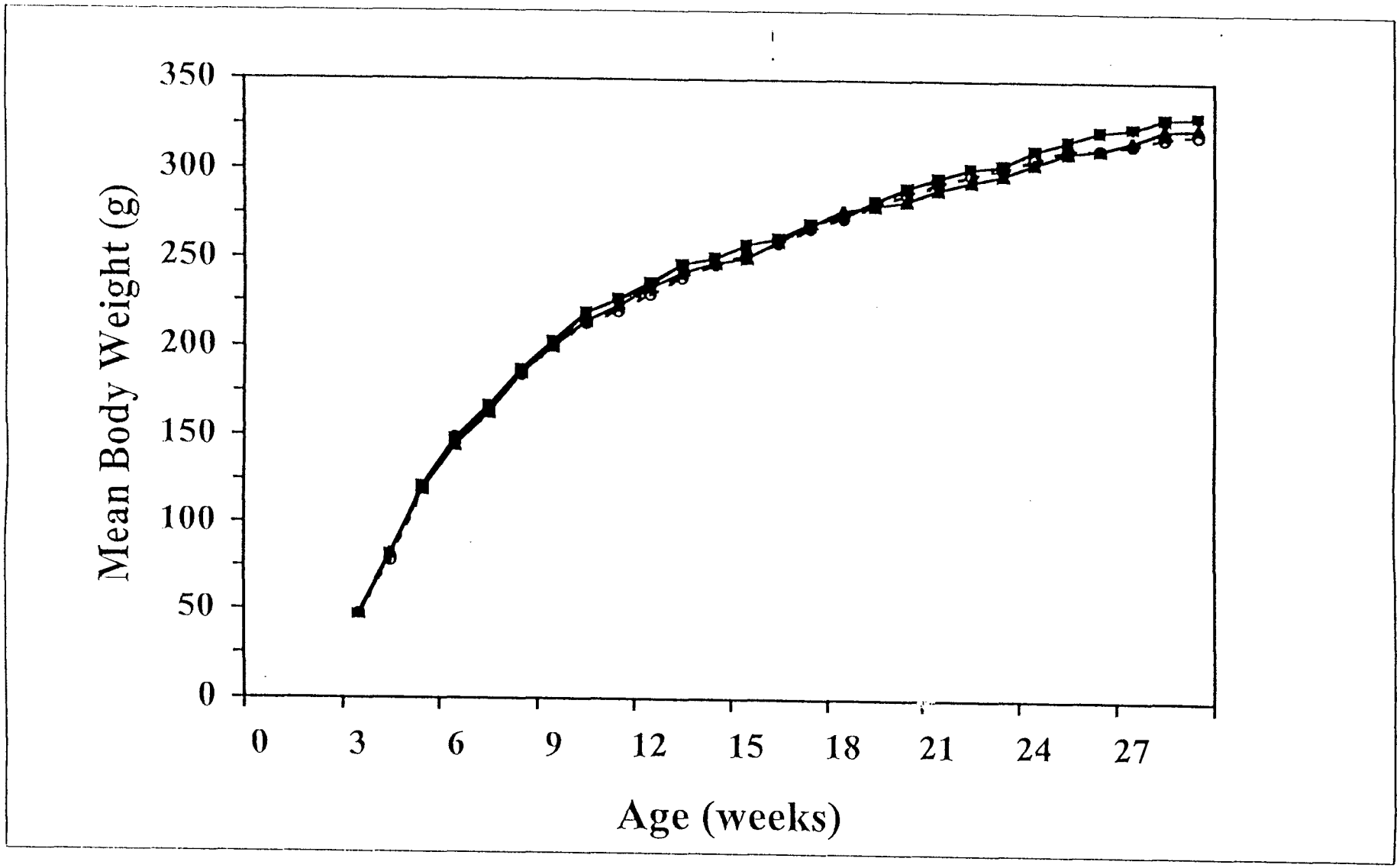
1. Results are expressed as mean ± SEM. Means in a row with different letters significantly differ at P=0.02.

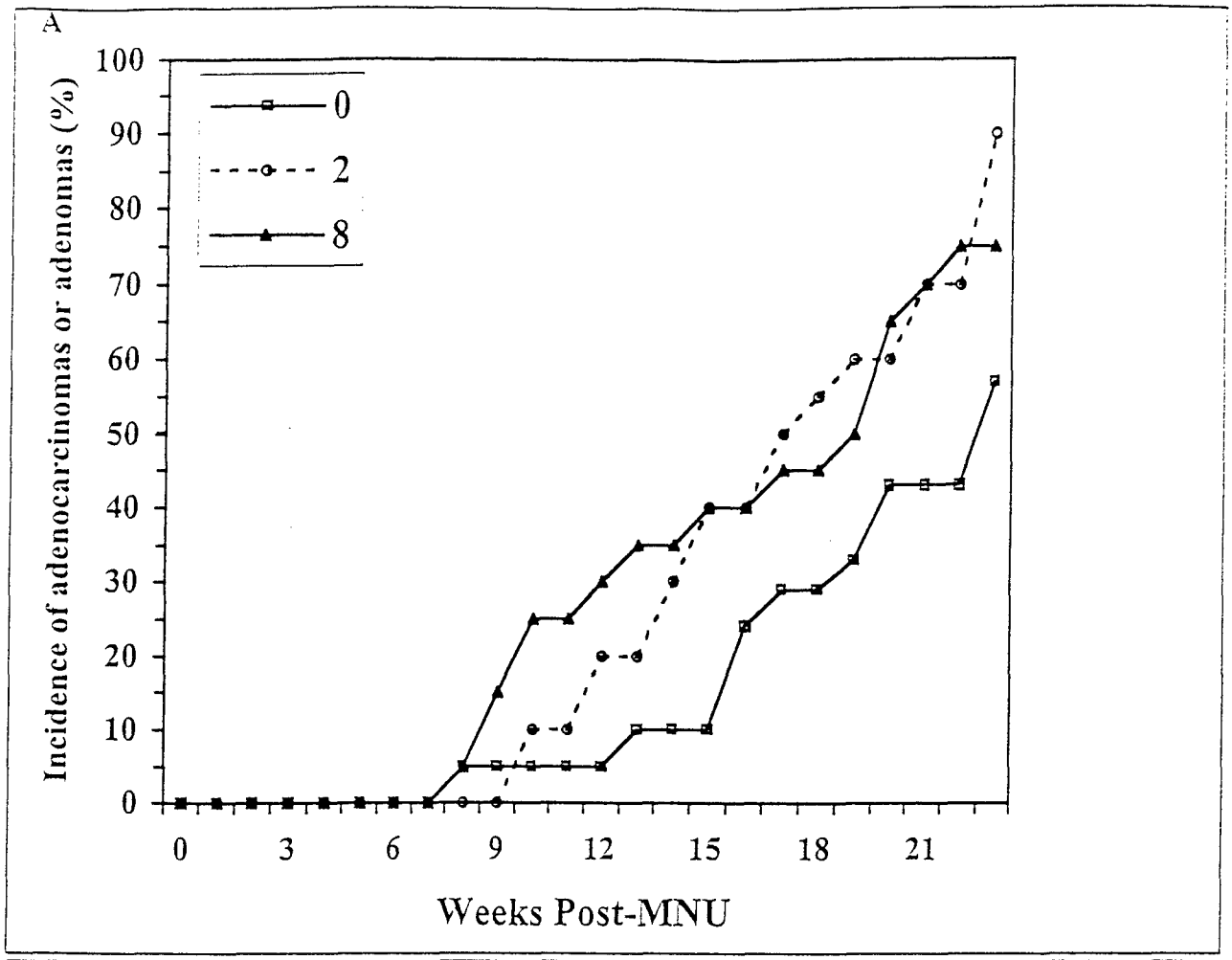
* Excluding 1 outlier in the 0 mg folate group, which harbored a total of 9 adenocarcinomas and adenomas, strengthened the overall comparison of the incidence of adenocarcinomas and adenomas among the three groups. By contrast, no significant difference in mean latency, multiplicity, volume and weight of adenocarcinomas and adenomas was observed among the three groups whether or not the outlier was included or excluded in the analyses.

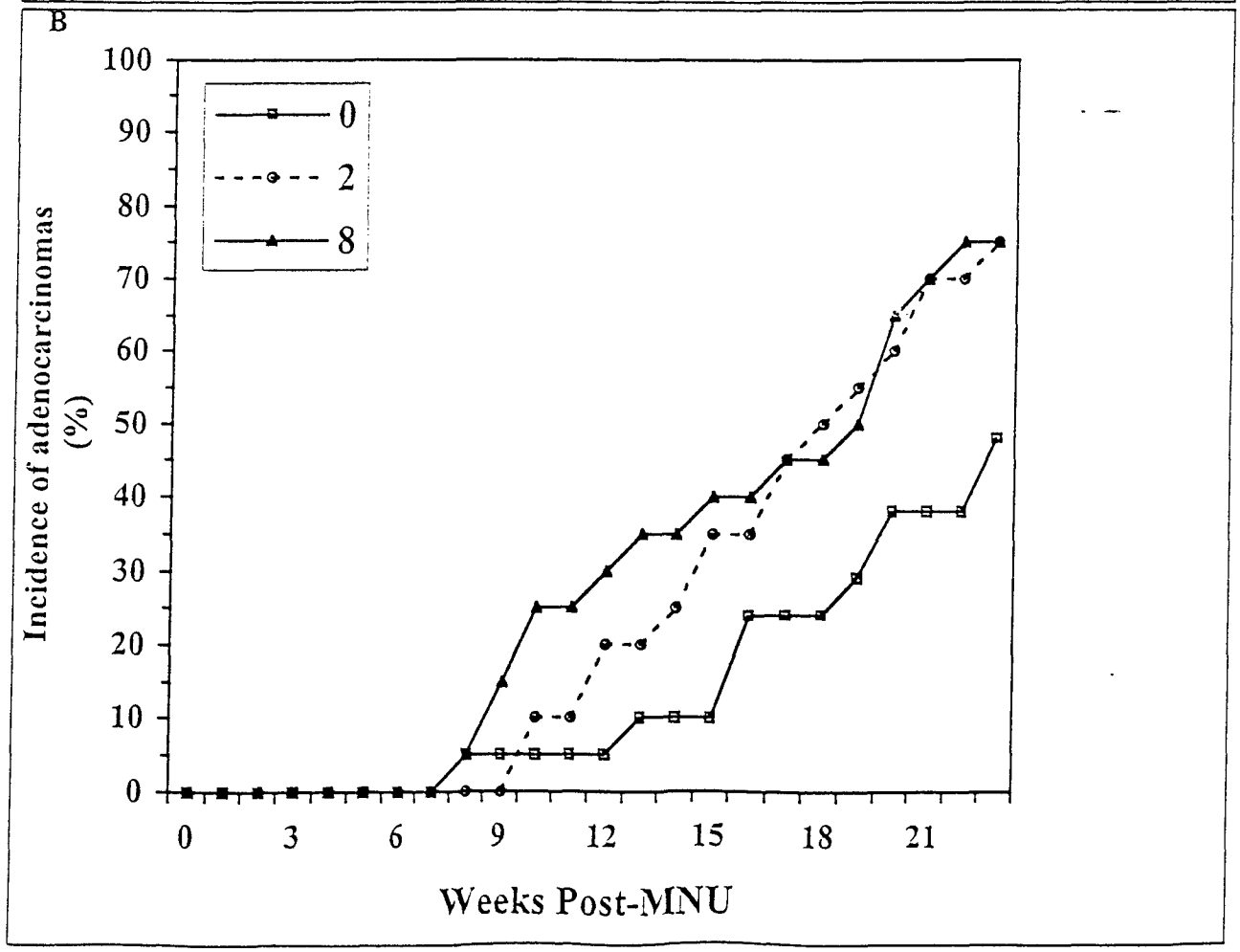
Table 3: Effects of dietary folate on the incidence, latency, multiplicity, volume and weight of mammary adenocarcinomas and adenomas[1]

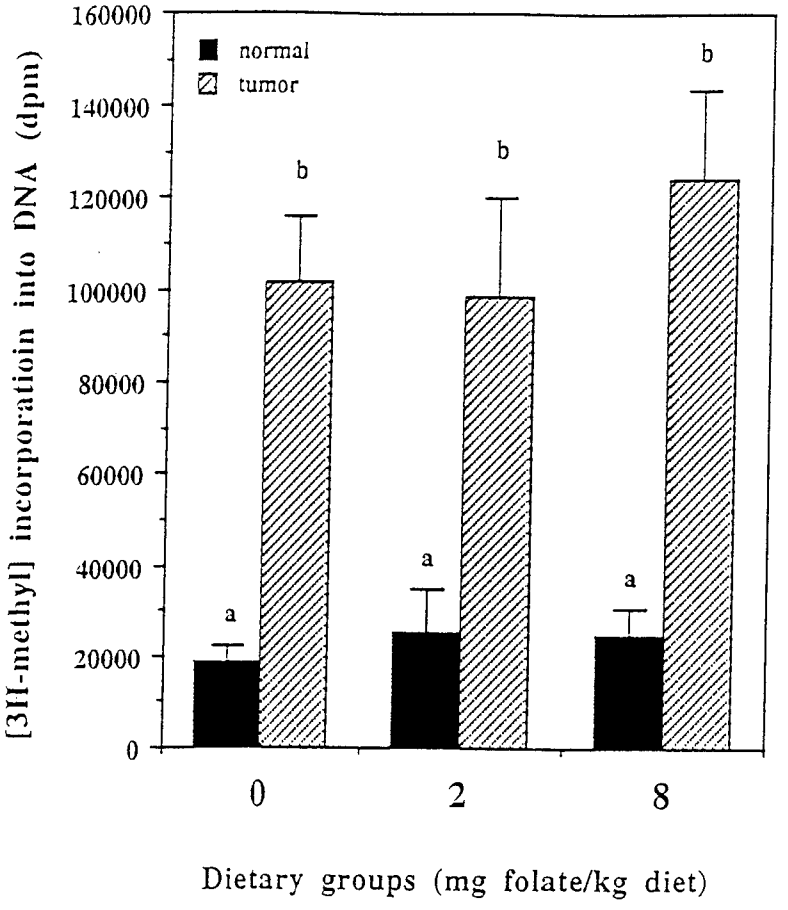
| Diet (mg folate/kg diet) | 0 | 2 | 8 | P-value |
|---|---|---|---|---|
| Incidence (%) | 48 | 75 | 75 | 0.10 |
| Mean latency (weeks post-MNU injection) | 17.40±1.54 | 16.27±1.10 | 15.00±1.36 | 0.51 |
| Mean multiplicity | 3.71±1.04 | 3.00±0.59 | 1.90±0.29 | 0.21 |
| Mean volme ($cm^3$) | 3.15±0.99 | 3.10±0.65 | 1.64±0.42 | 0.38 |
| Mean weight (g) | 0.98±0.28 | 1.12±0.25 | 0.63±0.15 | 0.42 |

1. Results are expressed as mean ± SEM. No significant difference in mean latency, multiplicity, volume and weight of adenocarcinomas was observed among the three groups.


Mean Body Weight (g)
350
300
250
200
150
100
50
0
0
3
6
9
12
15
18
21
24
27
Age (weeks)


A
Incidence of adenocarcinomas or adenomas (%)
0
2
8
0
10
20
30
40
50
60
70
80
90
100
0
3
6
9
12
15
18
21
Weeks Post-MNU


B
Incidence of adenocarcinomas (%)
0
2
8
0
10
20
30
40
50
60
70
80
90
100
0
3
6
9
12
15
18
21
Weeks Post-MNU


160000
140000
120000
100000
80000
60000
40000
20000
0
normal
tumor
[3H-methyl] incorporatioin into DNA (dpm)
a
b
a
b
a
b
0
2
8
Dietary groups (mg folate/kg diet)